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Acquisition and maintenance of specific bacterial symbionts in vertical and

horizontal symbioses

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ABSTRACT

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Symbionts, or microbial mutualists, can have profound effects on the ecology and evolution of multicellular organisms. These effects can differ in type or magnitude based on the genetic and phenotypic symbiont variation, so it is important to determine and understand the consequences of specificity in host-symbiont pairings. Using a framework wherein symbiont specificity can be determined at three points – in the external environment, within the host, and during transmission, I investigate how specificity can be imposed and the consequences of specificity in the host in vertical, or heritable, and horizontal, or environmentally acquired, symbioses. In the horizontal symbiosis between true bugs and *Burkholderia* bacteria, I show that specificity in *Burkholderia* acquisition is primarily driven by screening in the host, though external environmental conditions can determine the incidence of less prevalent *Burkholderia* in the host. I evaluated the location within the host where specificity is imposed by sequencing the bacterial communities in five distinct regions of the squash bug midgut using Illumina MiSeq. The vast majority of *Burkholderia* recovered throughout the midgut belonged to a single OTU, indicating that the host likely imposes specificity in a region anterior to the midgut. I used a vertical symbiosis, the pea aphid and its suite of facultative symbionts, to investigate how symbionts may promote their maintenance within the host by promoting aphid survival against parasitoid wasp attack. I found that one symbiont species, *Regiella,* enhanced the immune response used against parasitoids. The ability to enhance this immune response differed among *Regiella* strains, which may translate to differential maintenance of *Regiella* in host aphids. Finally, I evaluate symbiosis from the perspective of microbial symbionts, and suggest experiments and approaches that could incorporate symbiont fitness into investigations of symbiosis. The diverse approaches here, coupling phylogenetics, deep sequencing, and immunological assays highlight the challenges of developing a comprehensive understanding of the acquisition and maintenance of the world's diverse symbioses.

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Chapter 1

Introduction

All animals are colonized by microbes in some fashion. Some are transient and just pass through at the same rate as food particles. Others are specialized to certain host niches and are cultivated by the host. These microbes have important consequences for host ecology and evolution. Rhizobial bacteria, for example, can increase the growth of their leguminous host plants by 10-fold (Sachs *et al.* 2010a) and *Phyllodesmium* sea slugs can survive solely on the photosynthate provided by their symbionts (Burghardt *et al.* 2008). Symbionts (here considered as beneficial microbes that are maintained by hosts) can also protect their hosts from pathogens and predators (Oliver *et al.* 2003; Scarborough 2005; Teixeira *et al.* 2008; Stabb & Millikan 2009; Haeder *et al.* 2009). Variation caused by presence or absence of symbionts or the harboring of alternative symbionts can represent an important non-genomically encoded source of variation influencing host fitness and evolution. Therefore, understanding symbiont acquisition and maintenance can provide insights into the ecology and evolution of the host, the symbiont, and their shared evolutionary trajectory.

There are two main symbiont transmission routes: vertical and horizontal. In many symbioses, symbionts are vertically transmitted from a parent (typically the mother) to offspring. Classic examples include the large number of insects and arthropods that harbor *Wolbachia* (Hilgenboecker *et al.* 2008), hard corals that host photosynthetic golden-brown algae (Baker 2003), and aphids and their bacterial

symbionts (the obligate symbiont *Buchnera aphidicola* and several well-studied facultative bacterial symbionts (Oliver *et al.* 2010)). Selection has presumably shaped the evolution of mechanisms to facilitate these transmission mechanisms, ensuring the passage of beneficial bacteria to offspring over the often long co-evolutionary history of these host-symbiont associations.

Horizontal transmission, on the other hand, involves acquisition from outside the host environment each generation. These symbioses are presumably more labile, with offspring potentially exposed to a novel symbiont pool every generation. Model systems of horizontal transmission include leguminous plants and rhizobial bacteria (Oldroyd *et al.* 2011) and the defensive symbiosis between Hawaiian bobtail squid and luminescent *Vibrio fischeri* (Nyholm & McFall-Ngai 2004). Many diverse host-associated microbiomes, such as those in the gut, are also horizontally acquired, though similarities between parental and offspring microbiomes do suggest a potential role for vertical transmission (Turnbaugh *et al.* 2008; Faith *et al.* 2013).

Because symbionts can provide important benefits that increase host fitness (Kikuchi *et al.* 2007; Ferrari & Vavre 2011), and because symbionts can vary in the type and level of benefit that they confer (Oliver *et al.* 2005; Sachs *et al.* 2010b), there is strong selective pressure for hosts to shape the acquisition and maintenance of specific symbionts. Some of these factors are shared across horizontal and vertically transmitted symbioses, while others are unique to each (Bright & Bulgheresi 2010)(Figure 1). Here, I overview these processes and contrast the two transmission routes. Taking a whole approach and considering each of these

processes is necessary to get a comprehensive picture of the ecology and evolution of symbiotic systems.

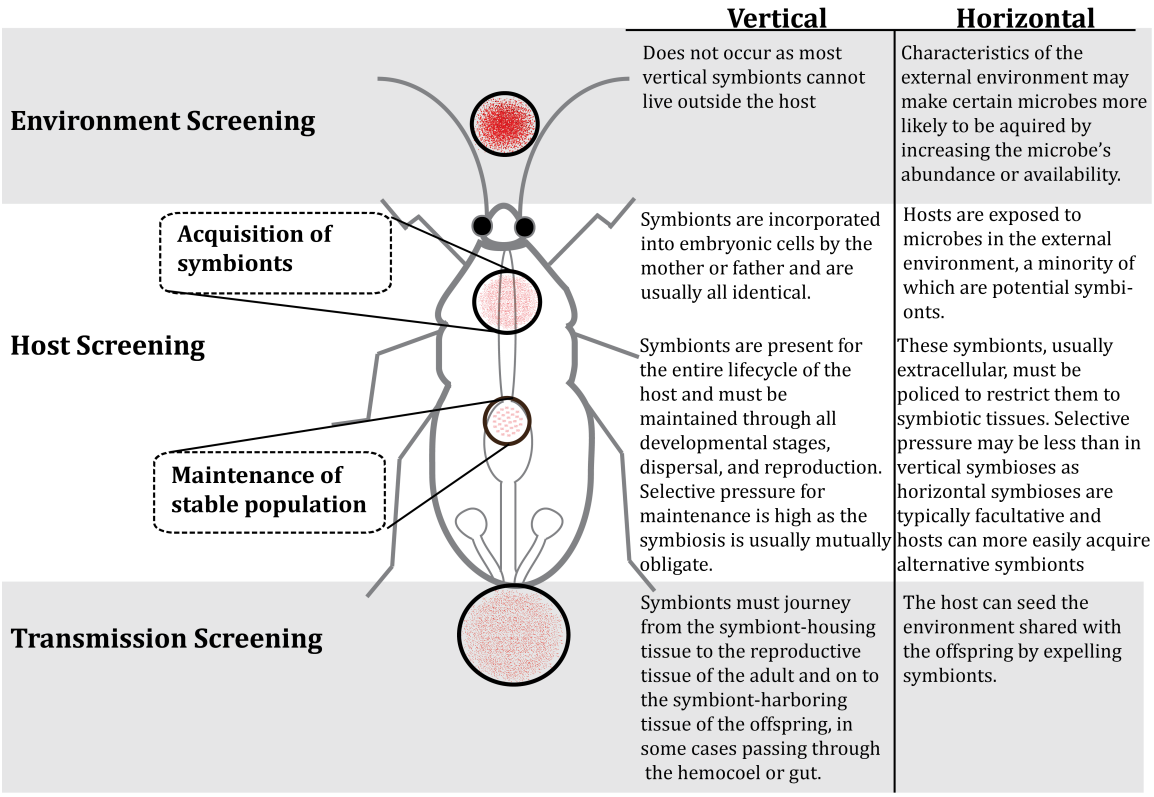

Figure 1-1. The three filters (environment, host, and transmission) where symbiont specificity can be determined in vertical and horizontal symbioses with characteristics typical of each transmission strategy described on the right.

Contact and acquisition

Recreating the endosymbiotic relationship in every generation represents a major hurdle for hosts in horizontal symbioses. Unlike their counterparts with vertically transmitted symbionts, hosts with environmentally acquired symbionts must contact, recognize, and capture the correct symbiont in environments that can host thousands of microbial species where the target symbiont is relatively rare. It is unclear whether symbionts can be detected and recognized through a secreted chemical signal or other cue under these conditions as any molecule would likely be extremely dilute in any environment that harbors symbionts (such as soil or seawater) or it could be difficult to distinguish it from the molecular signals of other microbes. However, hosts may excrete chemoattractants that assemble specific motile microbes near the host. For example, the bioluminescent bacterium *Vibrio fischeri*, a symbiont of Hawaiian bobtail squid and some marine fish, is found in seawater at a concentration of 100-1500 cells per milliliter of seawater, which translates to <0.1% of the bacterioplankton population in the host's habitat (Nyholm & McFall-Ngai 2004). Despite this scarcity, squid hosts rapidly distinguish *V. fischeri* cells at a concentration as low as 10 cells per milliliter from other bacterioplankton and chaperone them to a specific niche in the squid's mantle. It has been shown that *V. fischeri* preferentially aggregate in mucus on the mantle, but it is unknown if they are attracted to host cues (Nyholm & McFall-Ngai 2004). Alternatively, nymphs of the stinkbug *Megacopta punctatissima* exhibit a wandering behavior, presumably in search of symbiont capsules deposited by the mother, if they don't orally acquire their symbiont within an hour of hatching. In this case, the

nymphs could conceivably be attracted by a symbiont cue as symbionts are deposited at a high density and are sufficiently segregated from other microbes (Hosokawa *et al.* 2008).

Many hosts have a limited time to acquire their symbionts, after which they become refractory to symbiosis. The bobtail squid follows a program of symbiont acquisition that is shutdown after the symbionts emit a signal to indicate successful colonization of the light organ. This initiates an irreversible retraction of host structures used to acquire symbionts. If the symbiosis is lost after this point, or if retraction is signaled too early, it is highly unlikely that symbionts can be acquired (Nyholm & McFall-Ngai 2004). Western flower and onion thrips can only acquire their symbionts by feeding in the first larval stage, and symbiont prevalence in adult thrips is consequently variable (0 – 80% of adults are infected) (de Vries *et al.* 2001; 2008; Chanbusarakum & Ullman 2008; 2009). Onset of symbiosis has not been studied in detail in many systems, but the consistent composition of the symbiont community is evidence that symbionts are not continually being acquired, though there do seem to be exceptions (Belda-Baillie *et al.* 1999; Little 2004). It is important to note that consistent composition may not be representative of a short limited window of acquisition but instead may indicate that the first symbionts to colonize a host have a competitive advantage over late arrivers.

After initial contact, symbionts must be allowed entry to the host and move to their niche within the host. Many hosts have specialized cells or organs where symbionts reside, and symbionts run the risk of being digested or cleared by the immune system as they move there. Additionally, the host cannot be permissive to

all microbes. The molecular dialogue between host and symbiont during acquisition is one of the most studied areas in symbiosis, and different mechanisms exist for acquiring specific microbes out of many. The canonical example is the acquisition of rhizobial bacteria by leguminous plants in which bacteria and plants exchange a number of molecular signals that result in root nodule formation and the intracellular uptake of rhizobia (Cooper 2007). This is a "screen-in" strategy; each partner must exhibit a series of signals indicating they are compatible for the symbiosis to form. This is also the general strategy deployed in the squid-*Vibrio* symbiosis and in a true bug, which requires symbionts to display certain cell wall components in order to proliferate and persist within the gut (Kim *et al.* 2013b). Other hosts employ a "screen out" strategy in which microbes not meeting certain criteria are blocked entry or eliminated from the host. For example, coral larvae can take up a number of algal symbionts via phagocytosis, but non-species specific symbionts are removed from the host, likely through apoptosis of the host cell containing the symbiont (Colley & Trench 1983; Dunn & Weis 2009). Acquired microbes may also be screened out via more passive mechanisms like a high gut pH or a lack of compatible food sources within the host niche. Despite these in-depth studies in some systems, it is not well understood how specificity between host and symbionts is produced in general, and investigation into acquisition in animals is lacking.

Maintenance

Although hosts and symbionts form a mutualistic relationship, their cooperation creates many potential inter-partner conflicts. Harboring symbionts can be costly for hosts when resources are allocated to symbionts instead of self-growth, which can result in reduced lifespan, reproduction, or growth (Vorburger & Gouskov 2011; Laughton *et al.* 2014). In turn, symbionts, much like pathogenic microbes, must grow to a population sufficiently large to ensure transmission without growing so large as to trigger immune elimination or become virulent to the host. Successful management of these conflicts can lead to a long-lived and evolutionarily stable relationship between the host and symbiont. Breakdown in conflict management can lead to failure of the relationship, often with disastrous consequences to both partners. One dramatic example is coral bleaching, in which elevated temperatures or other stressors lead to increased production of harmful metabolites by symbionts, and the coral host reacts by expelling or degrading most or all of its symbionts (Weis 2008). However, a number of conflict management strategies have been investigated that result in long-term reciprocal benefit.

Unsurprisingly, the host immune system, which recognizes and regulates pathogenic microbes, was first hypothesized to play a role in conflict management between hosts and symbionts (McFall-Ngai 2007). In vertical symbioses, symbionts are typically sequestered in specialized symbiont-harboring cells or tissues, which shields them from the bulk of the immune reactions in the hemocoel. However, these cells or tissues can have specific, localized immune reactions. For example, there is increased expression of one specific antibacterial peptide, coleoptericin, in

the symbiont-harboring organ (bacteriome) of grain weevils that prevents the symbiont from escaping the bacteriome (Anselme *et al.* 2008; Vigneron *et al.* 2012). This peptide arrests symbiont cell division after DNA replication, preventing symbiont population growth and resulting in bacterial gigantism, which is hypothesized to make the symbiont more amenable to host control (Login *et al.* 2011). Lysozymes play a similar role in policing the symbionts in aphid bacteriocytes (Nakabachi *et al.* 2005)

The immune system also plays a role in regulating extracellular symbionts in horizontal symbioses (Ryu *et al.* 2010), and it may also help to maintain or establish partner specificity in these relationships (Nyholm & Graf 2012). The bean bug, which harbors symbionts of the bacterial genus *Burkholderia* in a specialized posterior section of the midgut called the crypts, expresses a lysozyme in the midgut before symbionts are acquired, possibly as a "screen out" mechanism to help establish the specificity of the interaction (Futahashi *et al.* 2013). However, this bug also produces antimicrobial activity against the specific symbiotic *Burkholderia* in the midgut section immediately anterior to the crypts (Kim *et al.* 2013a). This not only suppresses the symbiont population, but also keeps it sequestered to a specific tissue. In contrast, immune cells in the bobtail squid become tolerant to *V. fischeri* symbionts after colonization and, instead of functioning to suppress symbiont populations, seem to play a role in maintaining specificity as they can clear a range of non-symbiotic bacteria (McFall-Ngai *et al.* 2010).

In addition to immune-based mechanisms of conflict management, many hosts with horizontal symbionts seem to have other tools for regulating symbiont

populations. Expulsion or degradation of symbionts is one of these mechanisms. Bobtail squid, as mentioned above, do not seem to suppress their symbiont population using the immune system but instead have evolved a diel cycle in which 95% of symbionts are expelled everyday at sunrise, allowing the remaining 5% to re-establish a large symbiont population by nightfall. Other marine hosts routinely expel or digest their algal symbionts (McCloskey *et al.* 1996; Dimond & Carrington 2008), and there is some evidence that expulsion is targeted at specific symbionts with undesirable traits (McCloskey *et al.* 1996; Sachs & Wilcox 2006). For example, the upside-down jellyfish can preferentially expel symbionts with high growth rates, which can take excess host resources (Sachs & Wilcox 2006). Similarly, plants can "sanction" underperforming mycorrhizal symbionts by withdrawing or decreasing the resources allocated to the roots that host poor symbionts (Kiers *et al.* 2011). These mechanisms stabilize mutualisms between hosts and bacteria by ensuring the host is interacting with the most productive partner and maintaining that specificity throughout the host's life.

Transmission to the next generation

Transmission to the next generation is what separates and defines vertical and horizontal symbioses. Hosts with vertical transmission differ markedly in their physiological adaptations to facilitate transmission. Some vertical symbionts (most notably *Wolbachia*) are maintained in the germ line for the entire life of the hosts, negating any need for symbiont translocation within the host's body (Serbus *et al.* 2008). Other symbionts must undertake a sometimes circuitous journey from the

symbiont-housing organ or cells to the host's reproductive tissue in the adult and onwards to colonize the symbiont-housing organ of the offspring, during which selectivity can be imposed (Bright & Bulgheresi 2010).

In some insect hosts, symbionts are sorted into two populations– one destined for the symbiont-bearing tissue and another for the reproductive tissues or germ line cells. In some lice, symbionts are divided early in host development, with one population of cells moving to the gut where the symbiont-harboring organ develops and another moving to temporary storage vesicles in the hemocoel, which eventually degrade and release the symbionts to invade germ line cells in the nearby ovarioles (Frank 1996). Planthoppers have separate symbiont-housing organs for each of three symbiont species, with further sequestration within each organ of symbionts that are dedicated to the adult host and others that are transmissible forms that migrate to the rectum where they are passed on to the germ line (Frank 1996; Bressan & Mulligan 2013). The symbionts destined for the germ line and somatic cells differ greatly in their morphology. Somatic cell symbionts exhibit gigantism and are surrounded by degenerating symbiont cells, while germ line symbionts have a more typical size and shape (Bressan & Mulligan 2013).

In other hosts, symbionts must move from the symbiont-bearing tissue to the reproductive tissue. In pea aphids, the bacteriocytes that house the obligate symbiont *Buchnera* are located near the ovaries, but *Buchnera* must be exocytosed from the bacteriocytes, travel extracellularly to the reproductive tissue, and be endocytosed into the forming embryo. Although *Serratia*, a facultative endosymbiont in pea aphids, enters the forming embryo in the same way and in the

same location as *Buchnera*, an unknown cellular mechanism screens *Serratia* cells out of the primary embryonic bacteriocytes and sequesters them into a syncytium that eventually forms into sheath cells, which primarily house facultative symbionts (Koga *et al.* 2012). In this way, the aphid host imposes a selective transmission screen between obligate and facultative symbionts in the embryo and maintains a cellular tropism in the symbionts that is beneficial to the host, as *Buchnera* populations can be suppressed with negative consequences for the host when *Serratia* cells can invade the primary bacteriocytes (Koga *et al.* 2003).

Evolutionary selection for the maintenance of symbioses

In addition to the proximate causes of specificity that operate over the lifespan of a host discussed above, there are a number of ultimate, or evolutionary, phenomena that select for adaptations to acquire, maintain, and transmit symbionts. The most widely accepted and tested explanation for the persistence of mutualistic symbioses is the accrual of fitness benefits to the host or mutual fitness benefits to the host and the symbiont. Fitness benefits as a reinforcer of symbiotic interactions is common to both horizontal and vertical symbioses, though the benefits accrued in each symbiosis differ somewhat.

Traditionally, the most well studied host-derived benefits of symbiont association have been nutrient provision to the host by the symbiont (Salem *et al.* 2014). Vertical symbionts often provide this type of benefit, producing nutrients their hosts are unable synthesize, and horizontal symbionts are expected to do so as well. Many insect symbionts produce amino acids (Akman Gündüz & Douglas 2009;

McCutcheon *et al.* 2009; Douglas 2011) and vitamins, especially B vitamins (Hosokawa *et al.* 2010; Michalkova *et al.* 2014; Salem *et al.* 2014), for the benefit of the host, and this nutrient supplementation is typically necessary for host survival or reproduction (Koga *et al.* 2007). Both horizontal and vertical symbionts can also provide a second type of benefit - defending their host against pathogens and predators (Stabb & Millikan 2009; Dong *et al.* 2009; Krediet *et al.* 2013; Woodhams & Brucker 2013). The classical example of host defense by a horizontal symbiont is the bobtail squid – *Vibrio fischeri* symbiosis, in which bioluminescent *Vibrio* harbored in the squid's mantle provide counterillumination to camouflage the squid from predators at night (Stabb & Millikan 2009), though symbionts provide defense in a variety of ways including toxin or antimicrobial production (Haeder *et al.* 2009; Oliver *et al.* 2009) and immune priming (Clay 2014).

Investigation of host fitness benefits can take many forms. The standard experiment involves rearing hosts with and without their symbionts, sometimes under multiple ecological conditions (see Chapter two). Nutrient provisioning is easy to detect in these experiment as hosts always need certain nutrients, but the conditions under which other host benefits would be detected, such as under heavy parasitism or complex partnerships, are not always easy to re-create in the lab, if they are even known at all. Genomics, transcriptomics, and metabolomics can help fill these gaps. It was determined that the fungus farmed by leaf-cutter ant, for example, "pre-digests" plant material provided by its ant hosts with a set of diverse lignocellulases using genome sequencing and metaproteomics (Aylward *et al.* 2013).

Overall, understanding the natural history and considering the ecological context in which a symbiosis occurs can drive hypotheses about symbiont-derived benefits.

It is important to note that benefits for the symbiont could also select for microbial adaptations to maintain mutualistic associations. Host-derived benefits for symbionts have been less well studied than those for hosts (see Chapter four), but can also include nutrient provisioning, especially of amino acids (Graf & Ruby 1998; Prell *et al.* 2010). Defensive symbioses may also be a way symbionts can ensure their own maintenance by keeping the host healthy, as a dead host is generally a bad host. However, symbioses may also be maintained without a benefit to the symbiont as the host may be able to enslave the symbionts for their metabolic capabilities (Prell *et al.* 2010), *e.g.* through kleptoplastidy of the symbiont's chloroplasts (Johnson 2011), or prevent the symbionts from being expelled (Mergaert *et al.* 2006)(see Chapter four for further discussion).

True bugs as a model for horizontal symbioses

True bugs are a diverse and speciose group of insects that undergo hemimetabolous metamorphosis that includes a variety of agriculturally, economically, and medically important insects. In the past ten years, this group has been found to host bacterial symbionts that are acquired from the environment in contrast to most other insects studied to date (Salem *et al.* 2015), which largely host vertically transmitted symbionts or have a diverse consortium of gut bacteria that is more transient than a typical symbiont community. This system therefore provides

an unprecedented opportunity to study the acquisition and maintenance of a specific set of bacteria from the environment.

Many stinkbugs (encompassing true bugs in the order Heteroptera) host an environmentally transmitted bacterial symbiont in the genus *Burkholderia* within a specialized organ of the digestive tract called the midgut crypt (Kikuchi *et al.* 2005; 2007; 2011b). Most stinkbug hosts are aposymbiotic (without symbionts) at hatching and acquire *Burkholderia* symbionts from the soil as young nymphs (Kikuchi *et al.* 2011a). When not hosted by a stinkbug, the *Burkholderia* symbiont resides in the soil and the rhizosphere of leguminous plants and, unlike most other insect-associated symbionts, can be grown in the laboratory. This system is therefore ideal for investigating the ecology and evolution of horizontal symbioses because: 1) the stinkbug hosts can be naturally reared without *Burkholderia*, and 2) the *Burkholderia* symbiont can be grown in culture and introduced to the host. The ability to grow *Burkholderia* symbionts outside of the host and to establish specific infections within a host provides a unique opportunity to tease apart host and symbiont effects on specificity. Recent experiments have shown that the *Burkholderia* symbiont confers positive fitness benefits to some stinkbug host species; stinkbugs with *Burkholderia* symbionts are significantly larger or longer-lived than conspecifics without the symbiont (Kikuchi *et al.* 2005; Garcia *et al.* 2014) (Kikuchi *et al.* 2007). Although *Burkholderia* symbionts are present in many stinkbug species (Olivier-Espejel *et al.* 2011; Kikuchi *et al.* 2011b; Boucias *et al.* 2012), it is not known if all host-symbiont pairings are beneficial to the host or even if the midgut crypt is restricted to symbionts in the genus *Burkholderia*.

The pea aphid, *Acyrthosiphon pisum*, as a model of vertical symbioses

The pea aphid has become a canonical model for investigating symbiosis and host-microbe interactions. All pea aphids harbor *Buchnera aphidicola*, an obligate bacterial symbiont, in specialized cells called bacteriocytes. *Buchnera* provide pea aphids with amino acids that they cannot produce on their own and are not present in their food (Baumann *et al.* 1995), the nutritionally poor phloem sap of plants such as pea, fava bean, and clover. This endosymbiotic relationship is one of complete dependence. *Buchnera* has a drastically reduced genome and cannot live outside its host. Pea aphids have lower survival, less fecundity, and a smaller size in experiments where *Buchnera* has been experimentally cleared (Koga *et al.* 2003; 2007). *Buchnera* is transmitted to offspring matrilineally and has been continually present in pea aphids for 180 million years (Moran *et al.* 2008).

In addition, pea aphids can also host a number of "secondary" symbionts that are non-obligate but still beneficial. These symbionts can provide their pea aphid host with a variety of benefits including protection against pathogens (Oliver *et al.* 2003) and improved utilization of sub-optimal food sources (Leonardo & Muiru 2003). These symbionts are also vertically transmitted matrilineally, but transmission is not perfect and these symbionts can be lost from a population if ecological conditions change (Oliver *et al.* 2008). Additionally, these symbionts can cause deleterious effects including suppressing *Buchnera* populations (Koga *et al.* 2007) and increasing predation by ladybugs (Polin *et al.* 2014), so it may sometimes be beneficial to clear infections of secondary symbionts. These secondary symbionts

are ideal to investigate the factors that promote symbiont maintenance in a vertical symbiosis.

Overview of Dissertation

In this dissertation, I use both of these insect systems to investigate the establishment and maintenance of specific bacterial symbionts. In chapters two and three, I use stinkbug hosts to determine how specific their relationship with *Burkholderia* is by comparing bacterial communities within the host to those in its environment and comparing symbionts among four host species (Chapter 2). I show that symbiotic microbes in the midgut crypts of stinkbugs are a distinct subset of those found in the environment, suggesting the hosts can screen for specific bacteria. I then investigate where in the host this screening could take place using deep sequencing along the route of symbiont uptake in the host midgut (Chapter 3). *Burkholderia* incidence increases along the gut, but the whole midgut is colonized by highly similar *Burkholderia* that seem to be screened from other bacteria and *Burkholderia* strains before reaching the midgut.

In chapter four, I consider the microbial aspect of horizontal symbioses in a perspective piece that summarizes the literature on the fitness consequences of symbiosis on microbes. I suggest approaches and experiments to better test this.

Finally, in chapter five, I use the aphid system of vertical symbiosis to investigate how the maintenance of a facultative symbiont impacts a host immune response that may alter host resistance to natural enemies, thus altering the ecological and evolutionary pressures selecting for symbiont maintenance. One

facultative symbiont, *Regiella*, augments the melanization response in pea aphids, but there is some variation in the degree of augmentation across *Regiella* genotypes.

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Chapter 2

Partner associations across sympatric broad-headed bug species and their environmentally acquired bacterial symbionts

Modified from Garcia, JR, Laughton, AM, Malik, Z, Parker, BJ, Trincot, C, Chiang, SSL, Chung, E, and Gerardo, NM. 2014. Partner associations across sympatric broad-headed bug species and their environmentally acquired bacterial symbionts. *Molecular Ecology* 23 (6): 1333 – 1347.

ABSTRACT

Many organisms have intimate associations with beneficial microbes acquired from the environment. These host-symbiont associations can be specific and stable, but they are prone to lower partner specificity and more partner switching than vertically transmitted mutualisms. To investigate partner specificity in an environmentally acquired insect symbiosis, I used 16S rRNA gene and multilocus sequencing to survey the bacterial population in the bacteria-harboring organ (crypts) of 49 individuals across four sympatric broad-headed bug species (*Alydus calcaratus*, *A. conspersus*, *A. tomentosus* and *Megalotomus quinquespinosus*). Similar to other insect–bacteria associations, *Burkholderia* spp. were the most common residents of the crypts in all four insect species (77.2% of recovered sequences). *Burkholderia* presence was associated with prolonged survival to adulthood in *A. tomentosus*, suggesting a beneficial role of these specialized associations. *Burkholderia* were also found in environmental reservoirs in the insects' habitat, which may facilitate acquisition by insects by increasing *Burkholderia*–insect encounters. Symbiont establishment could also be facilitated by

resistance to insect defenses; zone of inhibition assays demonstrated that *Burkholderia* and other bacteria isolated from crypts are resistant to insect defenses that limit growth of *Escherichia coli*. Alternatively, the insects' defenses may not efficiently kill a broad range of bacteria. Although the symbiosis is targeted to *Burkholderia*, the insects' crypts housed other bacteria, including non-*Burkholderiaceae* species. There is no significant effect of host insect species on *Burkholderia* distribution, suggesting a lack of strong partner specificity at finer scales. The presence of frequent partner switching between sympatric insects and their symbionts likely prevents tight co-evolutionary dynamics.

INTRODUCTION

Most animals host beneficial symbiotic microbes that are required for survival or provide key benefits under specific environmental or physiological conditions (McFall-Ngai *et al.* 2013). In vertical mutualisms, symbiotic microbes are passed from a parent, usually the mother, to offspring. Consequently, the symbiont genotype in offspring is largely determined by the symbiont genotype present in parents, frequently leading to a long history of co-diversification between host and microbe (Baumann *et al.* 1995). Conversely, horizontal mutualisms are open systems where offspring must acquire symbiotic microbes from the environment each generation. In these mutualisms, the symbiont genotype present in each host can be determined by a variety of host (Davidson *et al.* 2004; Troll *et al.* 2010; Sachs *et al.* 2010; Nyholm & Graf 2012), symbiont (Davidson *et al.* 2004; Ruby 2008; Troll *et al.* 2010), and environmental (Lee & Ruby 1992; Finney *et al.* 2010; Porter & Rice 2012) factors. In most systems, it is currently unclear how these variables interact to determine which symbiotic microbes are present in an individual host.

Several pentatomomorphan stinkbugs and their symbionts are ideal for investigating the determinants of partner specificity in hosts with horizontally acquired symbionts. Many stinkbugs in the Lygaeoidea and Coreoidea superfamilies harbor bacteria of the genus *Burkholderia* in midgut crypts, specialized structures of the gut (Fig. S1, Supporting information; (Olivier-Espejel *et al.* 2011; Kikuchi *et al.* 2011b; Boucias *et al.* 2012). Symbiont detection assays and rearing experiments in *Riptortus pedestris* (Kikuchi *et al.* 2007) and *Thasus neocalifornicus* (Olivier-Espejel

et al. 2011) indicate insects hatch symbiont-free and acquire *Burkholderia* from the soil or the rhizosphere later in juvenile development (Kikuchi *et al.* 2011a). It is likely that this mode of transmission has been conserved in other insects that harbor *Burkholderia* as phylogenetic analyzes suggest that there are no clear host-symbiont co-evolutionary patterns across the Lygaeoidea and Coreoidea superfamilies (Kikuchi *et al.* 2011b). It is unclear what each partner receives from this association, although one hypothesis is that *Burkholderia* provide nutrients lacking in the hosts' diet as one host, *Riptortus pedestris*, grows larger and heavier and has higher survival when it harbors *Burkholderia* (Kikuchi *et al.* 2007; 2012), and other hosts develop more quickly and live longer (Olivier-Espejel *et al.* 2011; Boucias *et al.* 2012).

A broad survey of *Burkholderia* from 39 stinkbug species from the pentatomomorphan infraorder indicate that all species are phylogenetically constrained to three clades largely composed of insect-associated *Burkholderia*. Symbionts differ between insect species, but no coherent pattern of host-symbiont co-association is apparent (Kikuchi *et al.* 2011b). There has not, however, been a broad survey of bacteria isolated from sympatric insect species collected from multiple locations, which could reveal host or geographical factors that may shape partner specificity.

I investigated this symbiosis in four sympatric broad-headed bug species, *Alydus conspersus*, *A. tomentosus*, *A. calcaratus* and *Megalotomus quinquespinosus* (order Hemiptera, superfamily Coreoidea, family Alydidae) from 12 meadows in the

Southeastern United States. First, to determine whether *Burkholderia* provide benefits to broad-headed bug hosts, as they do for other insects (Kikuchi *et al.* 2007; Olivier-Espejel *et al.* 2011; Boucias *et al.* 2012), I assessed developmental time and survival of *Alydus tomentosus* with and without *Burkholderia*. Then, to address patterns of insect-bacteria association across these sympatric species, I used 16S rRNA gene and MLST sequencing to identify bacteria found in individuals across multiple locations. To find potential environmental reservoirs for insect symbionts, I preferentially isolated and sequenced *Burkholderia* in the soil and in association with the insects' food plants (*Lespedeza* spp.). Finally, through zone of inhibition assays, I tested whether *Burkholderia* and other crypt-isolated bacteria were resistant to insect hemolymph-based defenses, one potential mechanism by which host factors could facilitate partner specificity.

MATERIALS AND METHODS

Isolation of bacteria and bacterial DNA from insects, soil and plants

Male and female broad-headed bugs were collected from 12 sites in Georgia and North Carolina, United States (Table 1) in 2010, 2011, and 2012 by hand or with nets. Insects were frozen immediately at -80 °C or maintained in plastic cages. Broad-headed bugs were kept on a 16 hr light: 8 hr dark cycle at 25 °C or 28 °C and fed *ad libitum* autoclaved *Lespedeza capitata* seeds and one of two liquid diets: (i) water with 0.5 g/L ascorbic acid or (ii) 0.5 g/L ascorbic acid, 0.25 g/L cysteine and

0.025% blue food coloring (added to easily identify the digestive tract in dissected

Table 2-1: Number of insect, *Lespedeza*, and soil samples collected across locations.

Collection Site Name	Location[a]	Acal[b]	Acon	Atom	Mquin	Les[c]	Soil[d]
Chattahoochee Hatchery (CFH)	Blue Ridge				1		
Clairmont Campus (CM)	Atlanta	4		1			
Frances Meadows Aquatics (FM)	Gainesville	4	1				
Houston Mill Rd. (HM)	Atlanta				1		
Joy Baptist Church (JOY)	Wiley	1		3			
Lab-bred (LAB)	NA				2		
Lake Chatuge (LC)	Hayesville, NC			1			
Limestone Pkwy. (LIME)	Gainesville	2		2			
Morningside Preserve (MNP)	Atlanta		4	1			
Skeenah Gap Rd. (SKEE)	Blue Ridge			1			
Snapfinger Rd. (SNAP)	Atlanta						5
Songbird Meadow (SM)	Stone Mountain	4	5	7		5	10
U.S. 441 (WY)	Wiley		3		1		
Total		**15**	**13**	**16**	**5**	**5**	**15**

[a] in Georgia, unless otherwise noted
[b]Acal = *A. calcaratus*, Acon = *A. conspersus*, Atom = *A. tomentosus,* Mquin = *M. quinquespinosus*, Les = *Lespedeza cuneata*
[c]Number of root nodules
[d]Number of 0.1 g sub-samples taken from larger samples

insects). Insects were killed and surface-sterilized in 70% ethanol for 4 min and then rinsed with sterilized water. Dissections were performed in one of two ways: (i) the abdomen was cut open using sterile technique, and midgut crypts were removed from the rest of the gut, briefly rinsed in 70% ethanol and placed in Carlson's solution [0.7% NaCl, 0.02% KCl, 0.02% $CaCl_2$ hydrate, 0.01% $MgCl_2$ hexahydrate, 0.02% NaH_2PO_4, 0.012% $NaHCO_3$, 0.8% glucose; (Mitsuhashi 2002)] or (ii) the entire abdomen was clipped with sterile micro-scissors from the thorax and placed in Carlson's solution. Tissues from non-frozen broad-headed bugs were homogenized with a sterile pestle and plated on Luria-Bertani (LB) media. Plates were incubated for 2 days at 27 °C, and nine random colonies were then sub-

cultured and identified by sequencing the 16S rRNA gene. Tissues from frozen broad-headed bugs were thawed and used for DNA extraction and cloning (see below).

Broad-headed bugs frequently feed on the seeds of bush clover legumes (*Lespedeza* spp.), although they have been reported to feed on additional food sources available in the natural mixed legume fields that they inhabit throughout North America (Schaefer 1980; Ventura *et al.* 2000). Because *Lespedeza* spp. can host a variety of *Burkholderia* spp. in nitrogen-fixing root nodules (Palaniappan *et al.* 2010) and other tissues (Compant *et al.* 2008), I surveyed the bacteria within the root nodules of this host plant to determine whether the plants could serve as a reservoir or alternative host for insect gut symbionts. Sericea lespedezas (*Lespedeza cuneata*), common host plants of broad-headed bugs in Georgia, were collected from Songbird Meadow, a site where many broad-headed bugs were collected. Plants were excavated with a shovel and individually transported in plastic bags. Dissections of the root nodules were performed immediately upon return to the laboratory, following previously established protocols (Sachs *et al.* 2009). Roots were separated from plants and washed with deionized water to remove loose soil. After drying, root nodules were cut from the roots with sterile tools and sterilized by washing in 5% bleach for 2 min. Nodules were then rinsed in distilled water, placed in Carlson's solution, homogenized with a sterile pestle and plated on LB media. An approximately 1 cm section of root adjacent to each nodule was also removed, sterilized, homogenized, and plated on LB media to serve as a negative control. If there was bacterial growth from a non-nodule root section, the

corresponding root nodule bacterial cultures were not used. Plates were incubated for 2 days at 27 °C, and four random colonies were then sub-cultured and identified by sequencing the 16S rRNA gene.

Bulk soil near *Lespedeza* spp. patches (~8–15 cm away from any plant tissue) was collected with a shovel and transported in individual plastic bags from two sites in Georgia where broad-headed bugs are common (Songbird Meadow and Snapfinger Drive). Each sample consisted of the top six inches of soil, which was coarsely homogenized in the plastic bag. Five 0.1 g subsamples were taken from each soil sample and were diluted in 1 mL sterile phosphate-buffered saline (PBS). Each subsample was vortexed vigorously for 2 min and briefly centrifuged in a micro-centrifuge. To increase the likelihood of finding *Burkholderia*, 50 μL of supernatant from each subsample was plated on selective glucose-based rhizobium-defined medium (Sachs *et al.* 2009) with 200 mg/L cycloheximide and 2 mg/mL crystal violet. Plates were incubated for 2 days at 27 °C, and unique colonies were then sub-cultured and identified by sequencing the 16S rRNA gene.

DNA from cultivated bacteria isolated from insects, soil and plants was extracted with the Qiagen DNeasy Blood and Tissue Kit from cultures grown in LB overnight following the manufacturer's protocol (lysis step 30 min to 16 h). In addition, for direct cloning of bacterial PCR products from insects, whole insect abdomens were surface-sterilized as above and placed in buffer ATL, frozen in liquid nitrogen and crushed with a sterile pestle before DNA extraction following the manufacturer's protocol, with the lysing incubation step performed overnight.

All DNA extracts were stored at -20 °C until further use.

Development and survival assays

The effects of natural *Burkholderia* infections were tested in *A. tomentosus* by comparing development time and lifespan of *Burkholderia*-positive (symbiotic) and *Burkholderia*-negative (aposymbiotic) individuals. Eggs were taken from a general laboratory stock population of mixed genotypes, in which adults were allowed to mate freely. Two hundred and four eggs were divided into two treatments: (i) a *Burkholderia*-positive treatment in which insects were housed with soil that had previously been found to harbor *Burkholderia* that was collected from Songbird Meadow during insect collections; and (ii) a *Burkholderia*-negative treatment in which insects were housed with autoclaved soil to rear them aposymbiotically. Insects were inoculated through exposure to soil instead of pure bacterial culture because exposure to soil would allow the broad-headed bugs to acquire 'preferred' species. Eggs for both treatments were sterilized in 70% ethanol for 2 min followed by 10% bleach for 2 min, rinsed in sterilized DI water, and dried on kimwipes. Each egg was placed individually in a sterile 3 cm x 9 7.5 cm x 9 7.5 cm plastic box with autoclaved *Lespedeza* seeds, a sterile sponge saturated with nutrient solution (filter-sterilized water with 0.5 g/ L ascorbic acid, 0.25 g/L cysteine and 0.025% blue food coloring) and the appropriate type of soil. Broad-headed bugs are hemimetabolous insects that go through five nymphal stages before reaching adulthood; nymphs molt between each instar and between the fifth instar and adulthood. Insects were

surveyed every day after hatching to identify changes in instar (indicated by the presence of exuvia) and for survival. The *Burkholderia* infection status of each broad-headed bug was confirmed by *Burkholderia*-specific PCR with a previously published thermocycling program and set of primers specific to the *Burkholderia* genus (Kikuchi *et al.* 2005) on DNA extracted from the crypts. When a sample had no amplification in this PCR assay, it was assumed to be symbiont-negative. When the PCR results conflicted with the *Burkholderia*-exposure treatment, insects were reclassified according to the PCR results (which occurred in 17% of insects).

The number of days between molts was analyzed using linear mixed-effects (LME) models with the lme function of the nlme package (Pinheiro *et al.* 2013) in R v.2.11. Days between molts were log-transformed and tested for normality to ensure they fit model assumptions. The model included the random effect of individual to account for repeated measures of the same individuals. *Burkholderia* infection status and developmental stage (instar) were treated as fixed effects. I derived a minimal model using stepwise term removal and determined the significance of each factor using likelihood ratio tests with the ANOVA function. Terms were retained if their removal significantly reduced the explanatory power of a model. Survival data were analyzed using nonparametric survival models with a COXPH distribution using the survival package (Therneau 2013) in R. Data were tested for proportional hazards and for nonlinearity to ensure that data fit model assumptions. A minimal model was derived using stepwise term removal, and models were compared using chi-squared tests.

16S rRNA gene sequencing and multilocus sequence typing

The 16S rRNA gene was used to survey all bacterial constituents in this study, facilitating comparison to previously sequenced insect symbionts (Kikuchi *et al.* 2011b). Amplicons were produced using the 5 PRIME MasterTaq PCR kit in 22 µL reactions containing 2.5 µL 5X MasterTaq Buffer with Mg^{2+}, 10 mM of each dNTP (GenScript), 5 µM each primer [27F: 3′-AGAGTTTGATCCT GGCTCAG-5′; and a slightly modified 1492R: 3′-GGYTACCTTGTTACGACTT-5′; the underlined base was modified from a T to a Y; (Lane 1991)], 5 µL 5X TaqMaster PCR enhancer, 1 unit Taq DNA polymerase and 2 µL genomic DNA. The 1492R primer was modified to better bind *Burkholderia* 16S rRNA gene sequences by reducing mismatches. A negative control was included for each PCR assay by replacing the DNA with molecular-grade water. This reaction was denatured at 94 °C for 2 min, then cycled for 35 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, followed by final extension at 72 °C for 3 min. Amplicons were purified using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's protocol. PCR products from cultivated bacteria were then sequenced directly. PCR products amplified from insect crypts and abdomens were cloned using the Original TA Cloning Kit (Invitrogen) according to the manufacturer's protocol. Ten white colonies were chosen randomly for sequencing in the forward direction with the 27F or M13F primer.

To determine whether 16S rRNA sequencing captured the diversity and relationships found using other gene sequences, a subset of the cultivated

Burkholderia species isolated from broad-headed bug crypts, including multiple representatives from OTU 2 (see Fig. 2-2), were further characterized using the ATP synthase (*atpD*), glutamate synthase (*gltB*), leader peptidase (*lepA*) and recombinase A (*recA*) genes based on a multilocus sequence typing (MLST) assay previously developed for the *Burkholderia cepacia* complex and other similar species (Spilker *et al.* 2009). PCR amplification and purification were conducted as above except 10 μM of each primer was used. All reactions were incubated as done previously (Spilker *et al.* 2009). Briefly, *atpD* was annealed at 56 °C, *gltB* and *recA* at 58 °C, and *lepA* at 55 °C. PCRs for all genes were cycled for 30 cycles. Amplicons were directly sequenced with the forward primer of each primer set.

Sequence assembly and alignment

Only 16S rRNA gene sequences that had at least 600 bases with Phred quality scores of 20 or greater were used in downstream analyzes. SeqMan Pro version 10.1.1 (DNASTAR) was used to remove vector sequence and trim read ends. Trimmed sequences were aligned to the standard Hugenholtz 7682 character alignment in Greengenes (DeSantis *et al.* 2006b) using the NAST aligner (DeSantis *et al.* 2006a). All sequences were identified with the Ribosomal Database Project, Greengenes and NCBI taxonomies in Greengenes Classifier. Sequences that were identified as *Burkholderiaceae* were imported into mothur (Schloss *et al.* 2009). Thirty-five sequences that were not complete between Greengenes alignment positions 204 and 5809 were removed from further analysis. This included

sequences that were sequenced in the reverse direction with the M13F primer. The remaining sequences, as well as *Burkholderiaceae* 16S rRNA gene sequences from other studies of true bug symbionts (refer to Table S1, Supporting information), were clustered into operational taxonomic units (OTUs) with the average neighbor algorithm at 97% similarity cut-off level. A heatmap was created using mothur with a linear scale.

All genes in the MLST scheme (*atpD*, *gltB*, *lepA*, *recA*) were fully sequenced with the forward primer and trimmed as above in SeqMan Pro. Because most of the publicly available *Burkholderia cepacia* complex MLST sequences were produced according to an earlier scheme that generated shorter gene fragments (Baldwin *et al.* 2005), the sequences produced in this study were trimmed by hand to the same length as those in the pubMLST database (http://pubmlst.org/bcc/) after they were aligned with MUSCLE.

Phylogenetic analyses

Separate phylogenies were reconstructed for representatives of all *Burkholderiaceae* 16S rRNA gene OTUs and for a subset of cultivated *Burkholderia* species based on MLST sequence data. Nucleotide substitution models and parameters for phylogenetic analysis were chosen using jModelTest (Darriba *et al.* 2012). Tree topology was explored using distance, parsimony, maximum-likelihood and Bayesian models in mothur, PAUP* (Swofford 2003), Garli (Zwickl 2006) and

Mr. Bayes (Huelsenbeck & Ronquist 2001). Tree topologies were largely consistent between the models, and a final, bootstrapped maximum-likelihood phylogeny is presented. The final 16S rRNA gene tree topology was a maximum-likelihood phylogeny generated using a general time reversible model with empirical base frequencies, gamma-distributed rate variation among sites and four substitution rate categories in Garli. Starting trees were obtained via stepwise addition. Likelihood bootstrap values are based on 500 bootstrap replicates run in Garli with a GTR+G model and default Garli parameters except for the following: genthreshfortopoterm = 5000 and treerejectionthresholds = 50. The final MLST tree topology, based on ML, was generated in Garli using a general time reversible model with empirical base frequencies, gamma-distributed rate variation among sites, a proportion of invariant sites estimated from the data and four substitution rate categories. Starting trees were obtained via stepwise addition. Likelihood bootstrap values are based on 1000 bootstrap replicates and run with the same parameters as in the 16S tree.

Statistical comparison across insect hosts and geographical locations

Community structure of the *Burkholderiaceae* 16S rRNA gene sequences from the four sympatric insect hosts was analyzed using the vegan package (Oksanen *et al.* 2013) in R. Permutational MANOVA (the adonis function in vegan) was used to test the effect of species and location on OTU presence and absence. The matrix consisted of each sample (individual bug) by OTU presence/absence for the

four broad-headed bug species in this study. The full model included species, location and a species–location interaction, and non-significant variables were sequentially removed to arrive at the final model, which only retained location as a significant variable. Permutational MANOVA was run using the Jaccard index, a robust method for binary data with uneven sampling, to calculate pairwise distances from the matrix. P-values were calculated from 10,000 permutations. The analysis was restricted to only sequences classified as *Burkholderiaceae*. Permutational MANOVA was not performed with *Burkholderiaceae* from insect species outside our study because individual sample information was not available for these samples.

Using the same set of *Burkholderiaceae* sequences as for the PERMANOVA analysis, analysis of molecular variance (AMOVA) in mothur was performed to test for significant symbiont genetic differentiation across the four sympatric insect species and separately across locations. In addition, two AMOVAs were performed using samples collected as part of this study and samples collected previously from four other insect species (Table S1, Supporting information); one tested the significance of insect species, the other tested the significance of location (North America versus Asia). Nineteen host species classified as 'others' in Fig. 2-3 (Kikuchi *et al.* 2011b) were not included in these analyses because there was only one sequence per species available. All AMOVAs were performed with sequences (from one or more libraries) pooled according to host species or location. Each AMOVA consisted of 10,000 iterations, and significance was determined after Bonferroni correction. Because the effort to culture bacteria from the various insect species was not even, only sequences derived through PCR and cloning from insect gut and crypt

tissues were included in these analyses.

Zone of inhibition assays

The susceptibility of multiple bacteria to defenses in the hemolymph of *Alydus* spp. was tested using zone of inhibition (ZOI) plate assays. As many components of the insect immune system [e.g. antimicrobial peptides (Lemaitre & Hoffmann 2007)] are induced in response to signals of infection such as cell wall components like peptidoglycan, the susceptibilities of bacteria to induced and constitutive host defenses were tested first. Twenty *Alydus conspersus* were split into two treatment groups: (i) immune-challenged (injected with 1 µL of 0.5 mg/mL E. coli-derived lipopolysaccharide (LPS), a bacterial cell wall component, dissolved in insect Ringer's solution [128 mM NaCl, 18 mM $CaCl_2$, 1.3 mM KCl, 2.3 mM $NaHCO_3$; (Mitsuhashi 2002)]; and (ii) no-stab controls. Immune-challenged insects were injected with LPS between the second and third abdominal tergites using a pulled glass needle and incubated under normal rearing conditions for 24 hr post-treatment. Control insects were handled in the same manner, but not stabbed, and incubated for 24 h. Hemolymph from insects in both treatments was extracted via a neck wound swabbed with 70% ethanol and immediately frozen until required (-80 °C). ZOI assays were carried out following (Moret & Schmid-Hempel 2000)Moret and Schmid-Hempel (2000). Briefly, samples were defrosted on ice, and 1 µL of hemolymph was plated into small wells punched into 1% LB agar saturated with either E. coli MG1655 or *Burkholderia* 11BH497 (species isolated from an *Alydus*

calcaratus crypt which groups in an insect symbiont clade) and incubated overnight at 37 °C and 27 °C, respectively. Plates were scored for inhibition 24 hr later. Wells with no inhibition were scored as zero. When inhibition was present, the radius and area of the zone of clearance were measured under a dissecting scope and calculated with ImageJ (Abràmoff & Magalhães 2004).

The susceptibility of E. coli and *Burkholderia* 11BH497 to immune defenses induced by E. coli-derived LPS and heat-killed *Burkholderia* was compared to ensure that the previous results were not specific to E. coli-derived LPS. Eighteen *Alydus* spp. were divided into two treatments: (i) injected with LPS as done above; and (ii) injected with heat-killed *Burkholderia* 11BH497. The heat-killed *Burkholderia* solution was made by growing a culture of *Burkholderia* 11BH497 in LB to $OD_{600} = 1$ ($\sim 10^8$ bacterial cells per ml), resuspending the culture in 50 µL of PBS, and autoclaving for 15 min. Injected insects were incubated for 24 hr, and extracted hemolymph was plated on E. coli and *Burkholderia* 11BH497 ZOI plates as above. Following this experiment, the susceptibility of five additional bacteria to induced hemolymph defenses of *Alydus* spp. was tested. The following additional bacteria were used in this assay: (i) *Cupriavidus* sp. BHJ32i (Fig. 2-2, Table S2, Supporting information), a bacterium that is less common but still found in broad-headed bug crypts; (ii) *Lactococcus* sp. BHJ32a, a non-*Burkholderiaceae* bacterium isolated from a crypt sample; (iii) *Burkholderia fungorum* str. Snap2c (OTU 21 in Fig. 2-3, Table S2, Supporting information), a bacterium isolated from soil, but not crypts; (iv) *Burkholderia* sp. Les1n2i (OTU 7 in Fig. 2-3, Table S2, Supporting information),

a bacterium isolated from a *Lespedeza* root nodule that clusters with insect symbionts; and (v) *Serratia marcescens* str. RHoD, a pathogenic bacterium of many insects. Sixty-five insects were injected with 1 µL LPS as described above and

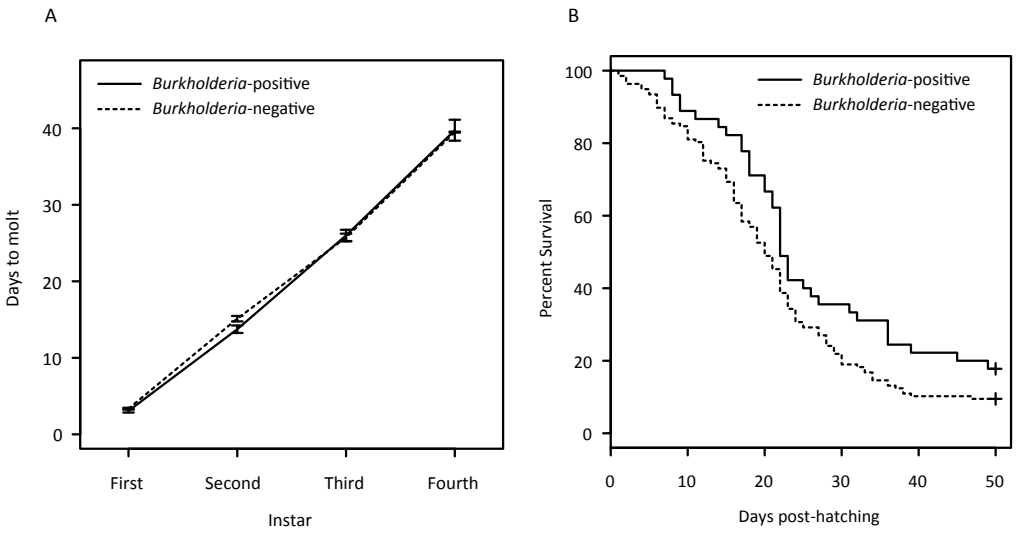

Figure 2-1. The effect of *Burkholderia* acquisition on *A. tomentosus*. A) Broad-headed bugs with *Burkholderia* have increased survival relative broad-headed bugs without *Burkholderia* (p = 0.036), but B) broad-headed bugs with and without *Burkholderia* have similar nymphal development times (p = 0.304). Error bars represent standard error.

incubated for 24 hr. Hemolymph was collected via a neck wound as above and then randomly assigned to one of the five bacteria listed above. In addition, three samples were assigned to an E. coli plate to ensure inhibition to this bacterium was present in these samples as before. Four samples were assigned to a *Burkholderia* 11BH497 plate to ensure there was no inhibition to this bacterium. When more than

1 µL was present in a sample, the remaining hemolymph was randomly assigned to another of the five bacteria plates. Phosphate-buffered solution (PBS) was added to one well on each plate to serve as a negative control. ZOI plates were incubated and scored as above.

RESULTS

Effect of Burkholderia on host survival and development

For *Alydus tomentosus*, the number of days between molts was significantly influenced by instar (p = 0.052), but not by *Burkholderia* infection status (p =0.304; Fig. 2-1A). However, *Burkholderia* infection significantly increased survival (χ^2 = 4.42, d.f. = 1, p =0.036; Fig. 2-1B). Infection with *Burkholderia* increased survival to adulthood in *A. tomentosus* by 2 days (*Burkholderia*-positive median lifespan = 22 days; *Burkholderia*-negative median lifespan = 20 days). Although we did screen every insect for the presence or absence of *Burkholderia*, we did not screen for other bacteria in the crypts, and *Burkholderia* may not be the only bacteria responsible for this effect.

Bacteria in insect crypts and abdomens

The majority of insect-associated bacterial sequences, both from crypts and whole abdomens, were bacteria in the *Burkholderiaceae*. A total of 183

Burkholderiaceae cloned sequences from 34 broad-headed bugs clustered into 18 OTUs (OTUs 1–13, 17–19, 29, 30; Figs. 2-2 and 2-3, Table 2-2; Table S1, Supporting information). Thirteen of the OTUs (1–13), which accounted for 90.8% of the sequences we recovered, grouped together in clades largely composed of *Burkholderia* spp. from Coreoidea and Lygaeoidea stinkbugs, which we refer to as the 'insect symbiont clades' (Figs. 2-2 and 2-3). Two OTUs (29, 30) closely related to *Cupriavidus* bacteria, another genus in the *Burkholderiaceae* family, accounted for 5.4% of recovered sequences. The remaining three OTUs grouped with other free-living or opportunistic pathogenic *Burkholderia* species and together accounted for 3.8% of the recovered sequences. More than half of the broad-headed bugs (19/34) were co-infected with two or more *Burkholderiaceae* OTUs (Table 2). Most of the co-infections (15/22) were composed of the most prevalent broad-headed bug OTU (OTU 2) and other less common OTUs. We used a MLST assay to characterize a subset of the bacteria for which we sequenced the 16S rRNA gene to ensure that the phylogenetic placement of OTU representatives was not specific to the 16S rRNA gene. Overall, the MLST OTU representatives grouped into the same clades as in the 16S rRNA gene phylogeny (Fig. S3, Supporting information).

Cultivation of bacteria from crypts resulted in bacteria similar to the cloned sequences. Twenty-five *Burkholderiaceae* bacterial sequences were recovered from seven individual insects (Table 2). These sequences fell into four major groups: the insect symbiont clade (with OTU 2), the *Burkholderia cepacia* complex (isolate BHJ35g), the plant-associated beneficial and environmental *Burkholderia* group (isolate BHJ34h) and *Cupriavidus* spp. (isolate BHJ32i) (Fig. 2-2).

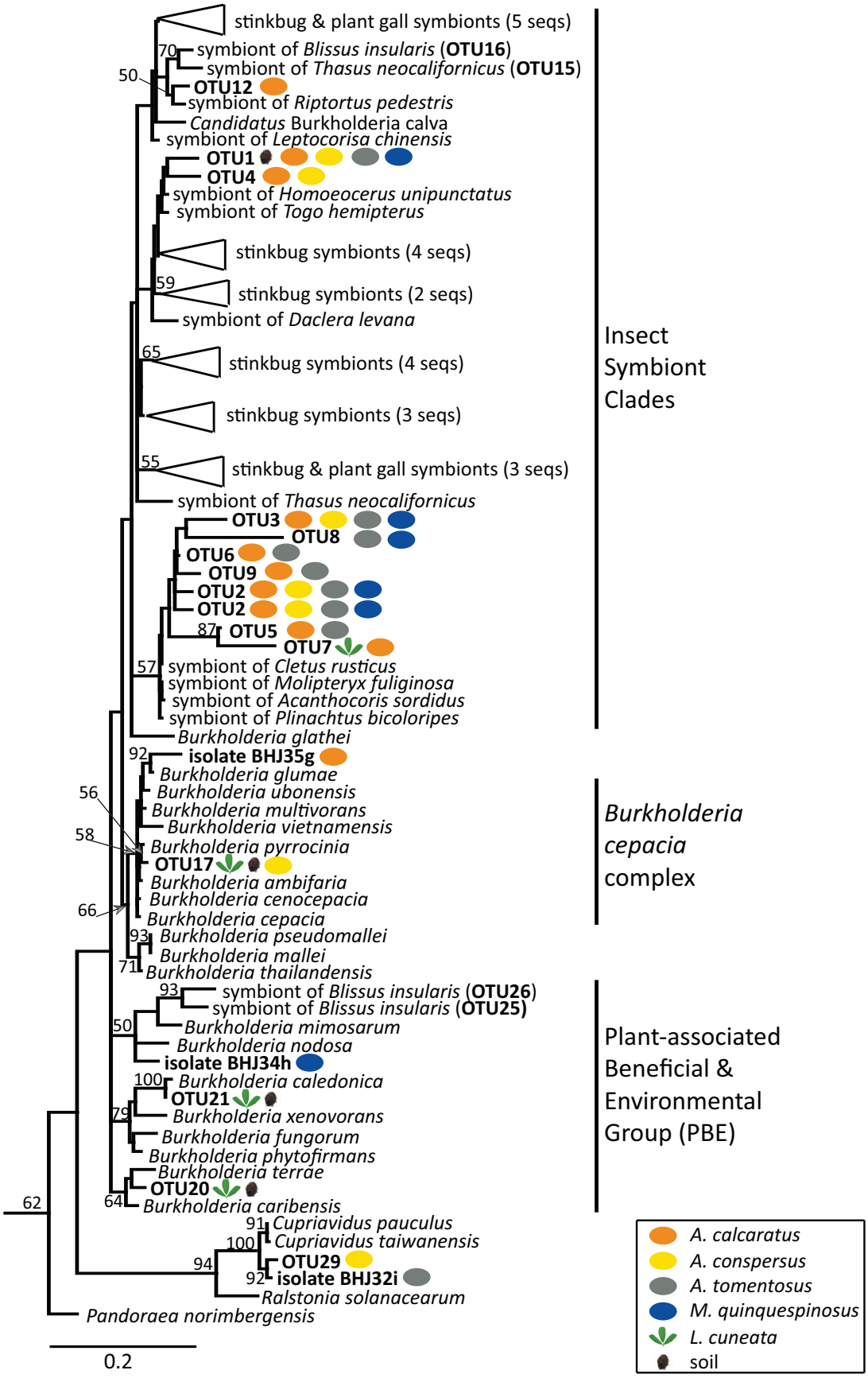
stinkbug & plant gall symbionts (5 seqs)
70
symbiont of Blissus insularis (OTU16)
symbiont of Thasus neocalifornicus (OTU15)
50
OTU12
symbiont of Riptortus pedestris
Candidatus Burkholderia calva
symbiont of Leptocorisa chinensis
OTU1
OTU4
symbiont of Homoeocerus unipunctatus
symbiont of Togo hemipterus
stinkbug symbionts (4 seqs)
59
stinkbug symbionts (2 seqs)
symbiont of Daclera levana
65
stinkbug symbionts (4 seqs)
stinkbug symbionts (3 seqs)
55
stinkbug & plant gall symbionts (3 seqs)
symbiont of Thasus neocalifornicus
OTU3
OTU8
OTU6
OTU9
OTU2
OTU2
87
OTU5
OTU7
57
symbiont of Cletus rusticus
symbiont of Molipteryx fuliginosa
symbiont of Acanthocoris sordidus
symbiont of Plinachtus bicoloripes
Burkholderia glathei
92
isolate BHJ35g
Burkholderia glumae
Burkholderia ubonensis
56
Burkholderia multivorans
Burkholderia vietnamensis
58
Burkholderia pyrrocinia
OTU17
Burkholderia ambifaria
Burkholderia cenocepacia
66
Burkholderia cepacia
93
Burkholderia pseudomallei
Burkholderia mallei
71
Burkholderia thailandensis
93
symbiont of Blissus insularis (OTU26)
symbiont of Blissus insularis (OTU25)
50
Burkholderia mimosarum
Burkholderia nodosa
isolate BHJ34h
100
Burkholderia caledonica
OTU21
79
Burkholderia xenovorans
Burkholderia fungorum
Burkholderia phytofirmans
Burkholderia terrae
OTU20
62
64
Burkholderia caribensis
91
Cupriavidus pauculus
100
Cupriavidus taiwanensis
94
OTU29
92
isolate BHJ32i
Ralstonia solanacearum
Pandoraea norimbergensis
0.2
Insect Symbiont Clades
Burkholderia cepacia complex
Plant-associated Beneficial & Environmental Group (PBE)
A. calcaratus
A. conspersus
A. tomentosus
M. quinquespinosus
L. cuneata
soil

Figure 2-2. Maximum likelihood phylogenetic analysis of the *Burkholderiaceae* 16S rRNA gene sequences, including all non-singleton OTUs in this study (in bold). Bold sequences labeled as "isolate" are bacteria cultivated from crypts. Circle, plant, and soil icons placed after each OTU number indicate where each OTU was recovered. Clades without OTU representatives from this study were collapsed. The tree was rooted with *Escherichia coli*. Numbers at nodes are ML bootstrap values (500 replicates). Scale bar at bottom represents 0.2 nucleotide changes per site. Bcc is an abbreviation for *Burkholderia cepacia* complex and PBE is an abbreviation for plant-associated beneficial and environmental *Burkholderia* group as previously outlined (Suárez-Moreno *et al.* 2011). A phylogeny with accession numbers for each sequence and without collapsed clades is available in the supplementary information (Figure S2).

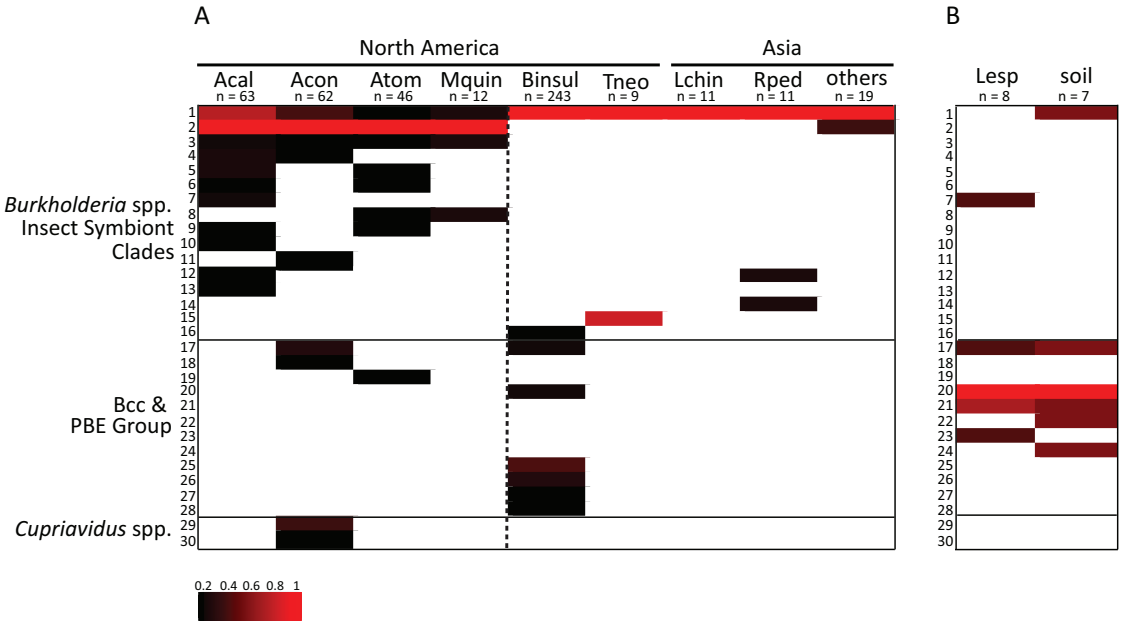

Figure 2-3. Heatmap of *Burkholderiaceae* sequence abundance. A) Sequences from cloned amplicons for the four sympatric broad-headed bug species from this study and 23 other insect species characterized in four previous studies. B) Sequences from direct sequencing of amplicons from bacterial cultures for the host plant *L. cuneata* and soil. Each numbered row represents an OTU defined at 97% similarity. The most abundant OTU is relativized to equal an abundance of 1. The abundances of the remaining OTUs are expressed as a percentage of the most abundant OTU. All species and sample types to the left of the dashed line are from this study; species to the right are from other studies as cited below. The black lines above the sample names indicate the location. Bcc is an abbreviation for *Burkholderia cepacia* complex and PBE is an abbreviation for plant-associated beneficial and environmental *Burkholderia* group as previously outlined (Suárez-Moreno *et al.* 2011). Acal = *Alydus calcaratus,* Acon = *Alydus conspersus*, Atom = *Alydus tomentosus*, Mquin = *Megalotomus quinquespinosus,* Binsul = *Blissus insularis* (Boucias *et al.* 2012), Tneo = *Thasus*

neocalifornicus (Olivier-Espejel *et al.* 2011), Lchin = *Leptocorisa chinensis* (Kikuchi *et al.* 2005), Rped = *Riptortus pedestris* (Kikuchi *et al.* 2005), others = pooled sequences of 19 Asian pentatomomorphan symbionts from a broad survey of symbiont diversity (Kikuchi *et al.* 2011b), Lesp = *Lespedeza cuneata* (bush clover), and soil = soil collected near *L. cuneata* plants. Sample information for sequences obtained from other studies is available in Table S1.

Eighty-five non-*Burkholderiaceae* bacterial sequences were recovered from the crypts and abdomens of the broad-headed bugs. The most abundant non-*Burkholderiaceae* bacteria were species in the α- and γ-subdivisions of the proteobacteria (Table 3). *Wolbachia* spp., common arthropod symbionts, and a *Rhizobium* sp. accounted for nearly all of the α-proteobacteria. The *Wolbachia* sequences were 99% similar to a number of bacteria isolated from ant-lions. The *Rhizobium* sequences were most closely related to a bacterium from the root nodule of a woody shrub (99% similarity), and most of the top BLAST hits (99–98% similarity) were bacteria isolated from plant or soil habitats. All of the γ-proteobacteria belonged to the *Chromatiaceae*, a family of anoxygenic photolithoautotrophic and sulphur-metabolizing bacteria.

Distribution of Burkholderiaceae *among insect hosts and across locations*

Three *Burkholderiaceae* OTUs (1, 2, 3) were shared among all the host insect species in this study (*Alydus* spp. and *M. quinquespinosus*; Fig. 2-3). All three of these OTUs fell within the 'insect symbiont clades', groups of bacteria that contain most true bug *Burkholderia* symbionts (Fig. 2-3). In addition, there were five OTUs that were shared between at least two of the host species from this study. Ten OTUs

were composed of sequences from only one insect species.

I used two approaches to detect differences between the symbionts distributed across the four sympatric host species and across meadows where they were collected. First, I tested for differences in community structure of *Burkholderiaceae* OTUs by running PERMANOVA on a sample X OTU incidence matrix. In this analysis, the final model retained only location as a significant variable (PERMANOVA, F = 1.78, d.f. = 8, $p = 0.009$). Host species was not significant (PERMANOVA, F = 1.59, d.f. = 3, $p = 0.078$). Second, I used AMOVAs, which test for differences in *Burkholderiaceae* genetic diversity as opposed to *Burkholderiaceae* OTU incidence, to test for differences based on host species and location. There was a significant difference in *Burkholderiaceae* genetic diversity across host species (AMOVA, F = 25.66, d.f. = 3, $p = 0.008$), but no significant difference across locations (AMOVA, F = -1.19, d.f. = 8, $p = 0.747$). The significant difference across host species was largely driven by the pairwise difference between *Alydus calcaratus* and *A. conspersus* (AMOVA, F = 53.79, d.f. = 1, $p = 0.007$).

The four broad-headed bug species and *Blissus insularis*, a species that has an overlapping range with the broad-headed bug species, have more symbiont diversity in comparison with other American and Asian Lygaeoidea and Coreoidea stinkbugs (Kikuchi *et al.* 2005; Olivier-Espejel *et al.* 2011; Boucias *et al.* 2012). AMOVA testing for differences in *Burkholderiaceae* genetic diversity across the species from this study and the previously investigated insects indicated a significant difference in *Burkholderiaceae* across host species (AMOVA, F = 17.85, d.f.

Table 2-2: *Burkholderiaceae* sequences found in insect, soil, and plant samples.

Species	Sample	Location	Method	Sample	No.	OTUs
A. calcaratus	11BH	CM	cultured	abdomen	1	n/a
	BHB6	SM	cloned	crypts	1	2
	BHJ35	LIME	cultured	crypts	1	n/a
	BHJ36	SM	cloned	crypts	4	2, 5
	C7	CM	cloned	abdomen	8	2, 3, 6
	C8	CM	cloned	abdomen	3	2, 10
	FMAC3	FM	cloned	crypts	8	1, 7, 12
	FMAC4	FM	cloned	crypts	9	2, 9
	FMAC6	FM	cloned	crypts	10	1, 4
	FMAC7	FM	cloned	crypts	10	1, 7, 13
	Joy1	JOY	cloned	crypts	6	2, 5
	Lime2	LIME	cloned	crypts	4	2
A. conspersus	3BH	SM	cultured	abdomen	2	n/a
	BHJ27	MNP	cloned	crypts	7	2, 17
	BHJ28	MNP	cloned	crypts	6	2, 17, 29
	BHJ29	MNP	cloned	crypts	7	2, 3, 29
	BHJ30	MNP	cloned	crypts	6	2, 29, 30
	FMAC8	FM	cloned	crypts	1	18
	SM13	SM	cloned	abdomen	3	1, 4
	SM2	SM	cloned	crypts	7	1, 2
	SM8	SM	cloned	crypts	7	1
	W3	WY	cloned	crypts	1	2
	W4	WY	cloned	crypts	6	2, 11
	W5	WY	cloned	crypts	11	2
A. tomentosus	2BH	SM	cultured	abdomen	3	n/a
	BHB5	SM	cloned	abdomen	3	2
	BHB7	SM	cloned	abdomen	1	2
	BHB11	SM	cloned	abdomen	2	2
	BHJ32	MNP	cultured	crypts	5	n/a
	BHJ38	SM	cloned	crypts	8	2
	BHJ39	SM	cloned	abdomen	10	2, 5, 8, 9
	ELF1	LC	cloned	crypts	1	2
	Joy2	JOY	cloned	crypts	1	1
	Joy3	JOY	cloned	crypts	9	2, 3
	Joy4	JOY	cloned	crypts	4	2
	Lime1	LIME	cloned	crypts	5	2, 6, 19
	Lime4	LIME	cloned	crypts	2	2, 8
M. quinquespinosus	BHJ23	LAB	cloned	crypts	2	1, 8
	BHJ31	HM	cultured	crypts	11	n/a
	BHJ34	CFH	cultured	crypts	2	n/a
	W2	WY	cloned	crypts	10	2, 3
L. cuneata	Les1	SM	cultured	nodule	1	7
	Les2	SM	cultured	nodules	1	17, 20, 21, 23
	Les4	SM	cultured	nodule	1	21
Soil	SML	SM	cultured	n/a	3	29, 20
	SMT	SM	cultured	n/a	1	22
	Snap	SNAP	cultured	n/a	3	1, 21, 24

Table 2-3: Percentages of sequences recovered from insect abdomens and crypts that were various non-*Burkholderiaceae*.

	Insect Species				Tissue Type	
Bacteria Species	Acal[a]	Acon	Atom	Mquin	abdomen	crypts
α-proteobacteria, *Bartonella* sp.[b]	2.2[c]					1.0
α-proteobacteria, *Brucella* sp.	2.2					1.0
α-proteobacteria, *Ochrobactrum* sp.	2.2					1.0
α-proteobacteria, *Rhizobium* sp.			2.8	13.3		3.1
α-proteobacteria, *Wolbachia* spp.		14.8	1.4	3.3	16.0	0.5
α-proteobacteria, *Sphingomonas* sp.		2.5			2.5	
β-proteobacteria, *Bergeriella* sp.	1.1		1.4		2.5	
γ-proteobacteria, *Allochromatium* sp.	1.1		2.8		3.7	
γ-proteobacteria, *Thiocapsa* sp.	4.3		1.4		6.2	
γ-proteobacteria, *Thiococcus* sp.	2.2				2.5	
γ-proteobacteria, *Thiodictyon* sp.	5.4		1.4		7.4	
γ-proteobacteria, *Thiorhodococcus* sp.	5.4				6.2	
Firmicutes, *Enterococcus* sp.	1.1		2.8		3.7	
Firmicutes, *Lactococcus* sp.			11.1		7.4	1.0

[a] Acal = *A. calcaratus*, Acon = *A. conspersus*, Atom = *A. tomentosus,* Mquin = *M. quinquespinosus*

[b] Identifications were made according to the NCBI taxonomy using Greengenes Classifier.

[c] Indicates the percentage of sequences including *Burkholderiaceae* and non-*Burkholderiaceae* sequences. I do not include any non-*Burkholderiaceae* sequences that were recovered only once.

= 7, $p < 0.0001$) and across continents (America vs. Asia; AMOVA, F = 14.11, d.f. = 1, $p = 0.049$).

Presence of Burkholderiaceae *in root nodules and soil*

The eight *Burkholderiaceae* sequences isolated from the root nodules of three *Lespedeza* plants clustered into five OTUs (7, 17, 20, 21, 23; Figs. 2-2 and 2-3). One sequence (OTU 7) fell within the insect symbiont clades, and the remaining four grouped with free-living and epiphytic *Burkholderia* species. The seven

Burkholderiaceae sequences isolated from soil clustered into six OTUs (1, 17, 20–22, 24; Fig. 2-3). One soil sequence (OTU 1) fell within the insect symbiont clades, and the remaining soil sequences grouped with *Lespedeza* root nodule sequences or with other *Burkholderia* (Figs. 2-2 and 2-3). Two OTUs were shared between plants and broad-headed bugs (OTUs 7 and 17) and between soil and broad-headed bugs (OTUs 1 and 17). Investigation of these sequences determined that while highly similar, the plant and bug sequences, as well as the soil and bug sequences, were not identical. Other non-*Burkholderia* bacteria were recovered from soil and root nodules, including *Rhizobium* spp., the typical root nodule symbiont for *Lespedeza* spp., but those data are not shown here.

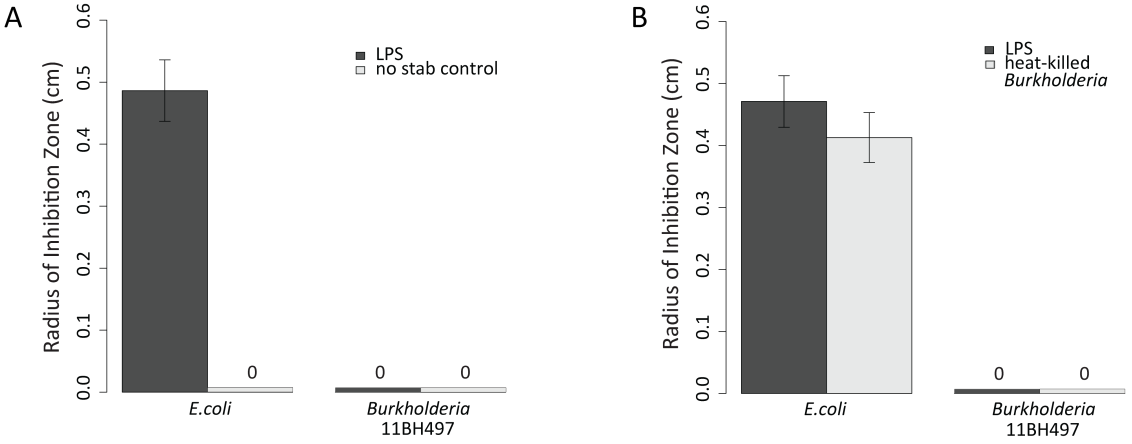

Figure 2-4. Susceptibility of *E.coli* MG1655 *and Burkholderia* sp. 11BH497 to *Alydus* spp. hemolymph defenses measured by zone of inhibition assays. A) Bacterial susceptibility to hemolymph defenses of insects injected with LPS or insects handled but not injected. B) Bacterial susceptibility to hemolymph defenses of insects injected with LPS or heat-killed *Burkholderia* 11BH4974. Error bars represent standard error.

Susceptibility of bacteria to hemolymph defenses

In a test of bacterial susceptibility to induced and constitutive immune defenses in *A. conspersus* hemolymph, *E. coli*, but not *Burkholderia* sp., was susceptible and only under the induced treatment (Fig. 2-4A). *E. coli* was not inhibited by non-induced host hemolymph, and *Burkholderia* was not inhibited by either hemolymph treatment (Fig. 2-4A). To ensure this result was not due to the activation of a limited repertoire of immune defenses by *E. coli*-derived LPS, the susceptibility of both bacteria was also tested with hemolymph from *Alydus* spp. that was stimulated with heat-killed *Burkholderia*. *E. coli* was similarly susceptible to hemolymph stimulated by heat-killed *Burkholderia* and *E. coli*-derived LPS. *Burkholderia* was not susceptible to hemolymph stimulated by either immune elicitor (Fig. 2-4B). The susceptibility of five additional bacteria (*Serratia marcescens*, *Lactococcus lactis*, a *Burkholderia* isolate from a root nodule, a *Burkholderia* isolate from soil (OTU 21) and a *Cupriavidus* isolate from crypts) was tested using LPS-stimulated hemolymph. Of these five bacteria, only the *Burkholderia* sp. isolated from a *Lespedeza* root nodule (OTU 7) was susceptible. This species was less susceptible than *E. coli* as evidenced by smaller zones of inhibition (Fig. 2-5). The remaining four bacteria were not inhibited by LPS-stimulated hemolymph.

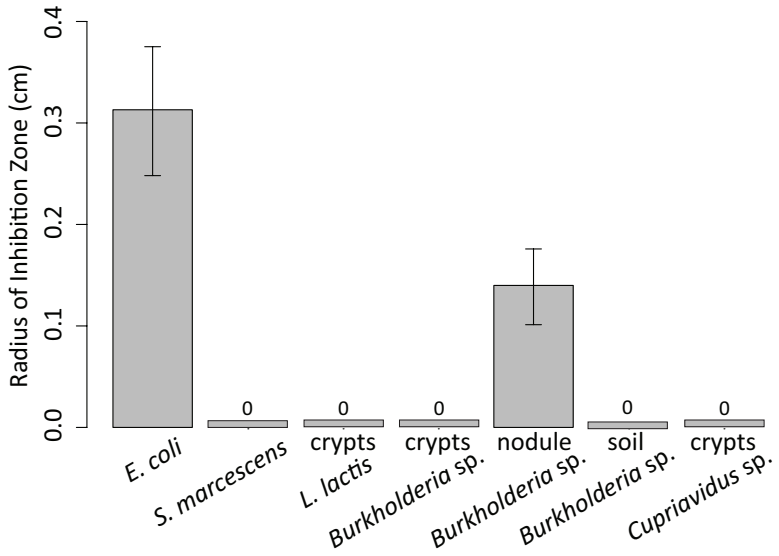

Figure 2-5. Bacteria differ in their susceptibility to LPS-stimulated *Alydus* spp. hemolymph defenses, as measured by zone of inhibition assays. Bacteria species are as follows (see Tables 2 and S1 for details of isolation): *E. coli* MG1655, *Serratia marcescens* RHoD, *Lactococcus lactis* BHJ32a, *Burkholderia* sp. 11BH497, *Burkholderia* sp. Les1n2i, *Burkholderia* sp. Snap2c, and *Cupriavidus* sp. BHJ32i. Error bars represent standard error.

DISCUSSION

Burkholderia spp. are the most frequent bacteria isolated from the midgut crypts of the four sympatric broad-headed bug species, similar to previously characterized bacterial communities in other hemipterans (Kikuchi *et al.* 2005; Olivier-Espejel *et al.* 2011; Boucias *et al.* 2012). Associations with gut symbionts can beneficially impact hemipteran development, growth and survival (Fukatsu & Hosokawa 2002; Douglas 2009; Prado & Almeida 2009). Specifically, establishment of *Burkholderia* in midgut crypts decreases development time and increases adult size and survival in *R. pedestris* (Kikuchi *et al.* 2007; 2012). In *Thasus neocalifornicus*, *Burkholderia*

acquisition increases nymphal survival (Olivier-Espejel *et al.* 2011), and in *Blissus insularis*, antibiotic clearance of *Burkholderia* retards development and increases mortality (Boucias *et al.* 2012). Here, there were no differences in development time between *Burkholderia*-positive and *Burkholderia*-negative *A. tomentosus*, but acquiring *Burkholderia* increased survival. Although it was not possible to attribute the survival benefits solely to *Burkholderia*, it is consistent with the evidence presented here and in the previous studies. Differences in the measured benefits across these studies may be methodological, or they may be due to differences in the benefits that are conferred by different *Burkholderia* species or to intrinsic differences between host species. While fitness differences between *Burkholderia*-positive and *Burkholderia*-negative broad-headed bugs in these studies presumably are due to *Burkholderia*, the acquisition or maintenance of other microbes may have been impacted by the experimental conditions, and future studies should assess the effects of other gut microbes as well.

Ecologically similar, sympatric broad-headed bug species presumably are exposed to the same environmental microbes, but many factors could lead to host-symbiont partner specificity nonetheless. Specificity can be considered at a number of different scales. For example, I can ask whether broad-headed bugs as a whole preferentially associate with some families of bacteria over others. Then, at a finer scale, I can ask whether broad-headed bugs as a whole preferentially associate with some bacterial species over others. Finally, I can ask whether broad-headed bug species differ in their associations with bacterial species. Consistent with previous studies, a majority of bacteria sequenced from the broad-headed bug crypts were

Burkholderia (77.2% of sequences), suggesting some mechanism by which *Burkholderia* associations are more frequently established over associations with environmental bacteria of other genera, of which there can be many [e.g. more than a 1,000,000 bacterial species in a gram of soil (Gans & Wolinsky 2005)]. I did, however, uncover some *Burkholderiaceae* bacteria outside the genus *Burkholderia* (see below) and non-*Burkholderiaceae* as well. A majority of the non-*Burkholderiaceae* sequences were isolated from whole abdomens rather than from the symbiont-housing crypts in isolation, making it likely that these bacteria were in other abdominal tissues or, despite careful sterilization, present on the exterior of the insects. A few of the non-*Burkholderiaceae* sequences, however, were isolated from crypts. Some of these bacteria (e.g. *Wolbachia* sp. and *Thiodictyon* sp.) were recovered from multiple individuals and species indicating that these bacteria may be stable and recurring constituents of the insects' microbiomes, although perhaps they occur on the outside of the crypts rather than within. Co-infections with these other bacteria, although rare, could result in bacterial interactions that alter host or symbiont fitness (Goto *et al.* 2006; Jaenike *et al.* 2009). For example, *Wolbachia* growth is suppressed in *Drosophila melanogaster* in co-infections with *Spiroplasma* compared to *Wolbachia*-only infections (Goto *et al.* 2006).

Focusing more closely on specificity in relation to associations with *Burkholderiaceae*, a majority of microbes isolated from *Alydus* spp. and *Megalotomus quinquespinosus* were *Burkholderia* spp. similar to those isolated from other insect species (i.e. *Burkholderia* from the 'insect symbiont clades'; Figs. 2-2 and 2-3). One *Burkholderia* OTU (OTU 1), abundant in a number of previously investigated insect

species (Kikuchi *et al.* 2005; Olivier-Espejel *et al.* 2011; Kikuchi *et al.* 2011b; Boucias *et al.* 2012), was recovered from our focal species, although at a low prevalence compared to *Burkholderia* OTU 2, which is nearly absent from all other previously characterized insects that host *Burkholderia* in crypts. We also isolated *Cupriavidus*, another genus within the *Burkholderiaceae*, (Figs. 2-2 and 2-3) from *A. conspersus* and *A. tomentosus*. These bacteria have not previously been reported as insect symbionts and are typically associated with plants, especially *C. taiwanensis*, a widespread and dominant symbiont of *Mimosa* spp. root nodules (Chen *et al.* 2003). While we found a significant difference in *Burkholderiaceae* genetic diversity due to host species when we considered both American and Asian insect species, when we focused on only the four sympatric hosts, we found no significant impact of host species on *Burkholderiaceae* incidence, although there was evidence of significant differences in the genetic diversity of *Burkholderia* across these species, which was possibly driven by the isolation of *Cupriavidus* from only *A. conspersus*. These results suggest that these sympatric species encounter and then sequester similar symbionts. Significant differences between the symbionts present in insects from different meadows does suggest the potential for different host-symbiont dynamics across natural habitats, although this may be weak as there was no difference in genetic diversity across habitats.

Many insects were co-infected with two or more *Burkholderia* OTUs (Table 2). Co-infection is present in *Riptortus pedestris* and *Leptocorisa chinenesis* (Kikuchi *et al.* 2005), but at a much lower frequency than found in *Alydus* spp. and *M. quinquespinosus*. It is unclear whether mixed *Burkholderia* symbiont infections are

beneficial to the insect host. In most of the co-infections, the common *Burkholderia* sp. (OTU 2) was present, although it is not clear whether this species was at low or high abundance relative to its co-infecting species. Co-infecting species could represent cheaters (Sachs *et al.* 2010) or could represent symbionts that provide different benefits. However, co-infection could also lead to within-host competition that could have negative fitness effects for co-infected hosts (Sakurai *et al.* 2005; Oliver *et al.* 2006). Most animal guts are co-infected with many different bacterial species, and the interactions between these species likely have important implications for both the host and the bacteria. Broad-headed bug-*Burkholderia* associations provide insect systems in which to test these hypotheses.

Overall, symbiont diversity in broad-headed bug populations is greater than that in some previously studied species. I isolated *Burkholderia* species from *Alydus* spp. and *M. quinquespinosus* individuals that fell outside the *Burkholderia* clade dominated by insect symbionts (Figs. 2-2 and 2-3). The southern chinch bug also occasionally harbors these *Burkholderia* (Boucias *et al.* 2012), but neither the giant mesquite bug (Olivier-Espejel *et al.* 2011) nor 19 Asian insect species do (Kikuchi *et al.* 2011b). While this could be an artifact of sampling depth in some cases, extensive sampling in two Asian species, *Riptortus pedestris* and *Leptocorisa chinensis*, did not uncover any symbiont associations outside the 'insect symbiont clades' (Kikuchi *et al.* 2005). Therefore, greater symbiont diversity associated with some insect species compared to others could reflect ecological differences. For example, *R. pedestris* were collected from soybean fields (Kikuchi *et al.* 2007; 2011b), where agricultural practices in a monoculture setting may limit the diversity of bacteria present in the

environment or where there may be selection for a narrower range of symbiotic partners [e.g. those that provide insecticide resistance (Kikuchi *et al.* 2012)]. Within more diverse natural settings of mixed plant species, variation in symbionts, which could have alternative metabolic capabilities including detoxification of specific plant compounds or protection against more diverse pathogens, could select for the maintenance of symbiont species outside the insect symbiont clade. Further research on the maintenance of *Burkholderia* genetic and phenotypic diversity in alternative ecosystems is needed.

Despite the fact that I isolated diverse *Burkholderiaceae* and other bacteria from our focal species, several lines of evidence suggest that these broad-headed bugs, like other true bugs, preferentially associate with a subset of *Burkholderiaceae*. First, *Burkholderia* species isolated from *Alydus* spp. and *M. quinquespinosus* were concentrated in the 'insect symbiont clades' instead of being evenly dispersed across the *Burkholderia* phylogeny. Second, while a few *Burkholderia* OTUs were isolated from both broad-headed bugs and soil or plant tissues, many *Burkholderia* isolated from the broad-headed bugs' environment were not found within broad-headed bugs. These results, consistent with previous studies of some other insect-*Burkholderia* associations (Kikuchi *et al.* 2007), suggest a process in which the insects associate with only a subset of available *Burkholderia*.

I examined several factors that could facilitate such specificity. Maintenance of *Burkholderia* within other habitats or hosts, for example, could function as an ecological mechanism leading to more frequent insect encounters with

Burkholderia. I isolated the same *Burkholderia* OTU from both the broad-headed bug *Alydus calcaratus* and from rhizobium-harboring root nodules of its leguminous food plant, *Lespedeza cuneata*. It is unclear whether this *Burkholderia* species is a beneficial root nodule symbiont (*i.e.* a bacterium that provides fixed nitrogen to the plant), but *Lespedeza* does have an intimate relationship with this bacterium; multiple *Burkholderia* species have previously been recovered from *Lespedeza* spp. nodules (Compant *et al.* 2008; Palaniappan *et al.* 2010). Although Palaniappan et al. (2010) found that *Burkholderia* could not induce nodule formation in the absence of nodule-competent bacteria such as *Rhizobium*, *Burkholderia* did have plant growth-promoting properties. While it is unlikely that broad-headed bugs directly acquire bacteria from nodules, plant associations with *Burkholderia* could maintain a higher concentration of *Burkholderia* in the soil surrounding host plants. To determine whether these hosts are sharing a *Burkholderia* symbiont pool, however, further investigation is needed to determine whether *Burkholderia* species isolated from plants can establish and be maintained in insects and, similarly, whether plants can uptake *Burkholderia* isolated from insects.

Many host and symbiont traits could also facilitate partner specificity. Recent research, for example, suggests that in another true bug, *R. pedestris*, antimicrobial activity within a region of the gut adjacent to the crypts, but not within the crypts themselves, suppresses *Burkholderia* (Kim *et al.* 2013a). Furthermore, *Burkholderia* with loss of function mutations in the *uppP* gene, which is involved in biosynthesis of cell wall components that trigger host immune responses (Loutet & Valvano 2011), are able to colonize but not establish in the crypts of *R. pedestris* (Kim *et al.*

2013b). These findings suggest that host immune responses towards bacteria play a role in symbiont establishment and maintenance. To understand how bacterial growth could be suppressed elsewhere in symbiotic hosts, using traditional zone of inhibition assays, I found that bacteria exhibit different susceptibilities to induced defenses of the host hemolymph (Figs. 2-4 and 2-5). While *E. coli* was susceptible to inhibition, bacteria from four other genera were not. This is in contrast to most insects, where antibacterial activity after immune stimulation is strong, long lasting and general to broad categories of microbes [e.g. Gram-positive or Gram-negative bacteria; (Lemaitre & Hoffmann 2007; Haine *et al.* 2008)]. The limited antimicrobial activity seen in assays with broad-headed bugs suggests that they may have narrower antimicrobial responses than other insects.

While zone of inhibition assays indicated that a broad range of bacteria are resistant to broad-headed bug host immune factors, some *Burkholderia* species were resistant, while others were not. Specifically, a *Burkholderia* species isolated from *Lespedeza* and genetically similar to *Burkholderia* from insect guts was inhibited by host hemolymph, while other *Burkholderia* were not. These differences are likely due to differential susceptibility to host antimicrobial peptides, but could also be due to differential susceptibility to reactive oxygen species, phenoloxidase, or cellular defenses. Further work will be needed to understand whether susceptibility to hemolymph-based defenses in zone of inhibition assays is correlated with the ability to colonize crypts or maintain long-term associations with insects. Given that the four sympatric host species studied here harbor similar symbionts, the mechanisms underlying specific associations with particular

Burkholderia are likely universal.

Our goal was to sample symbionts from multiple individuals of sympatric insect species to determine whether fine-scale sampling could reveal partner specificity. While I hypothesized that ecological or physiological differences between sympatric hosts could lead to differences in their symbiont populations, I found no evidence for differences in their symbiont populations based on the incidence of particular OTUs and only limited evidence of differences in their symbiont populations based on genetic diversity. Lack of partner specificity across these hosts could be driven by similarities in behavior, host plant use or life cycle or by shared ancestry. Regardless of the reason, partner-switching likely prevents tight co-evolutionary dynamics between hosts and symbionts in this system.

ACKNOWLEDGEMENTS

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SUPPLEMENTAL INFORMATION

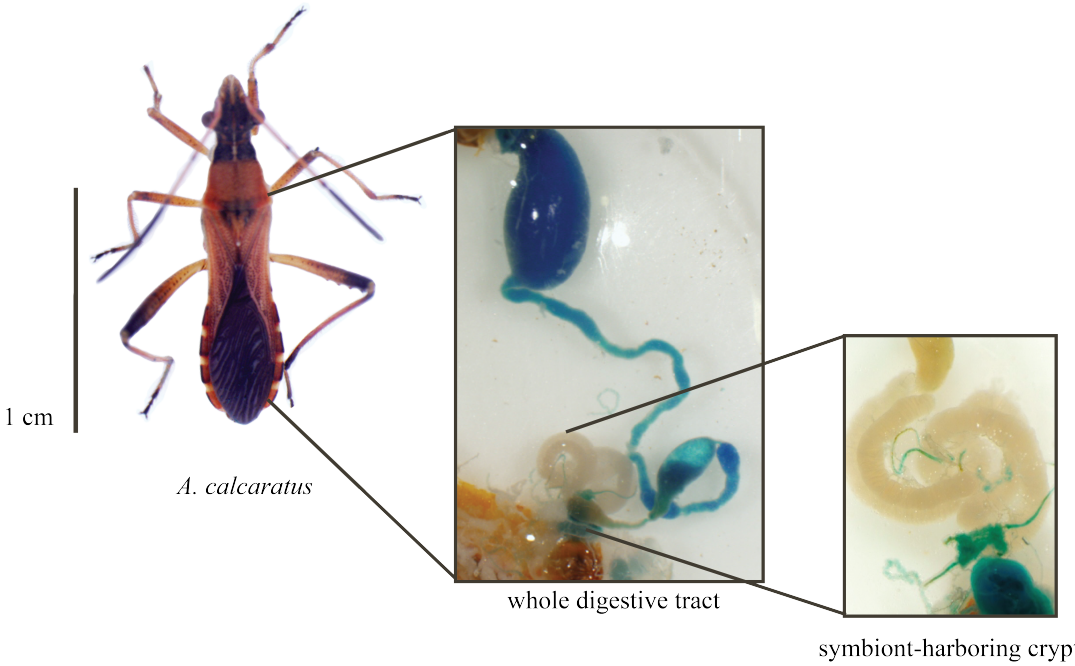

Figure S2-1: Picture of broad-headed bug midgut with detailed picture of the symbiont-harboring crypts. The digestive tract is artificially blue as food coloring was added to the vitamin- and amino acid-supplemented water in order to more easily identify the digestive tract during dissections.

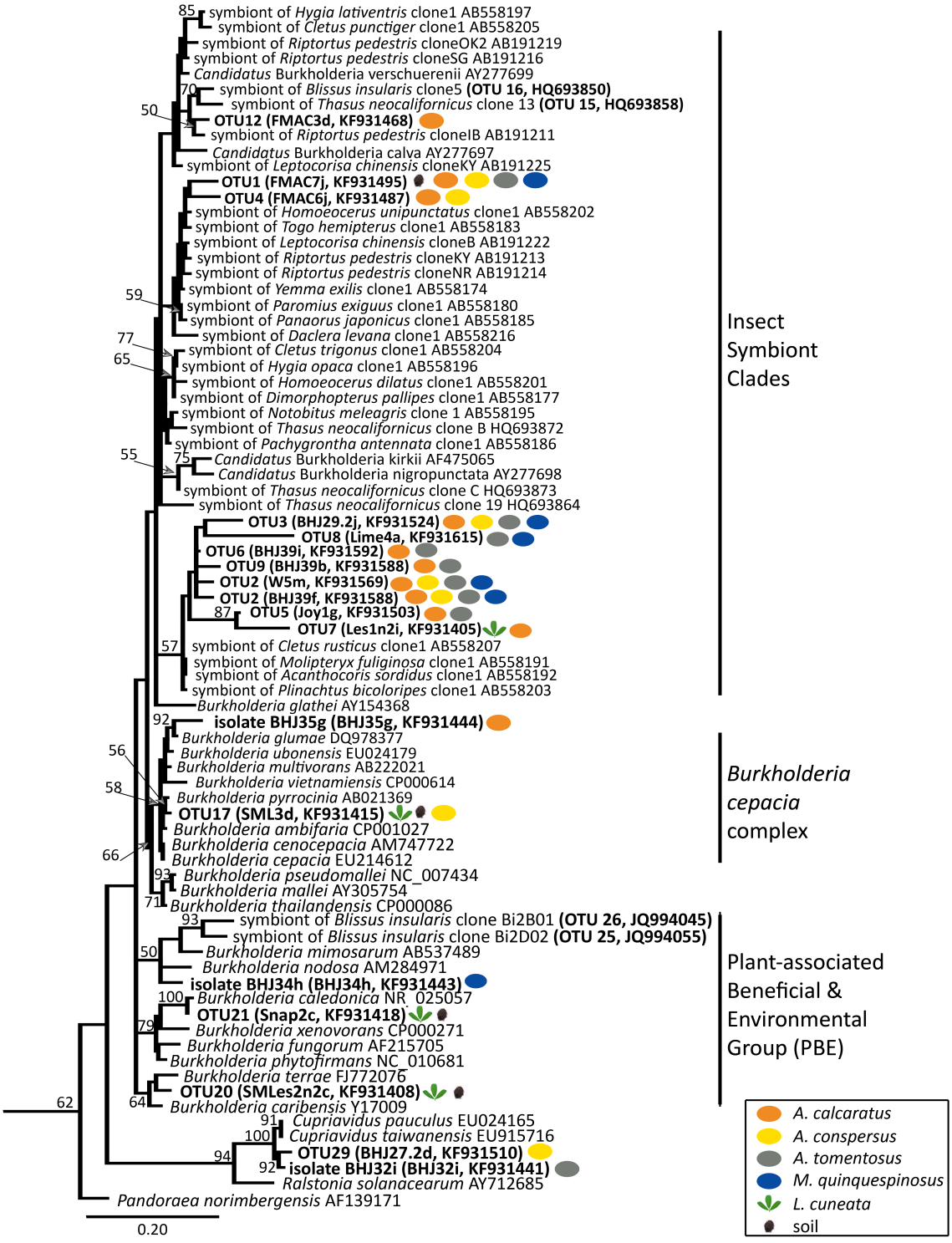

Figure S2-2: Phylogenetic analysis of the *Burkholderiaceae* 16S rRNA gene sequences from all non-singleton OTUs in this study (in bold). The sequenced representative of each OTU is in parentheses after the OTU number. Genbank accession number follow the name of each representative. The tree was rooted with *Escherichia coli*. Numbers at nodes are ML bootstrap values (500 replicates). Scale bar at bottom represents 0.2 nucleotide changes per site.

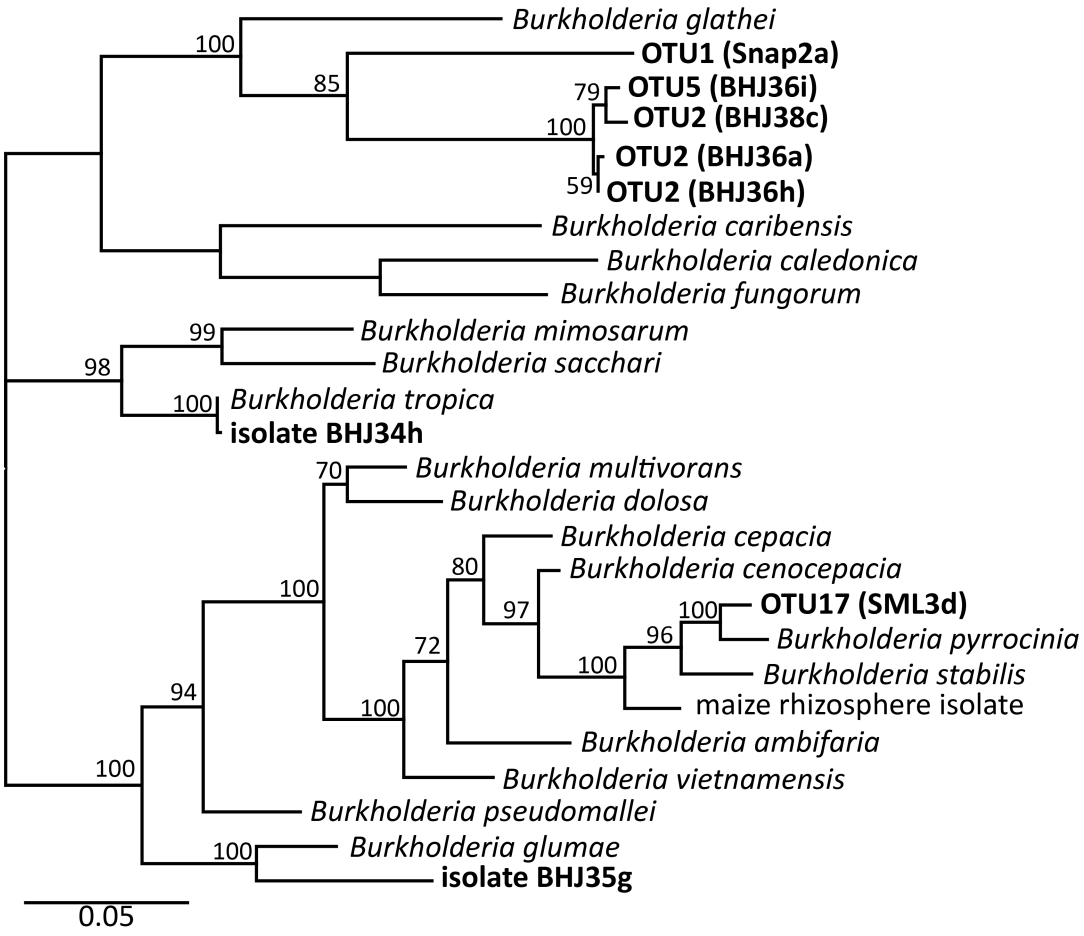

Figure S2-3. Phylogenetic analysis of eight cultivated *Burkholderia* species using a concatenation of four housekeeping genes: *atpD*, *gltB*, *lepA*, and *recA*. Sequences from this study are in bold with sequence names in parentheses. Numbers at nodes are ML bootstrap values (1000 replicates). Tree is unrooted. Scale bar at bottom represents 0.05 nucleotide changes per site.

Table S2-1: Sample information for *Burkholderiaceae* sequences obtained from previous studies.

Host Species	**n**[a]	**Organ**	**Method**	**Study**
Blissus insularis	243	crypts	cloning & rolling-circle amplification	(Boucias *et al.* 2012)
Leptocorisa chinensis	10	midgut & crypts	cloning & RFLP typing	(Kikuchi *et al.* 2005)
Riptortus pedestris	12	midgut & crypts	cloning & RFLP typing	(Kikuchi *et al.* 2005)
Thasus neocalifornicus	25	midgut	cloning & RFLP typing	(Olivier-Espejel *et al.* 2011)
"Others" from Fig. 3	19	crypts	cloning & RFLP typing	(Kikuchi *et al.* 2011b)
-*Yemma exilis*	1			
-*Dimorphopterus pallipes*	1			
-*Paromius exiguous*	1			
-*Togo hemipterus*	1			
-*Panaorus japonicas*	1			
-*Pachygrontha antennata*	1			
-*Molipteryx fulginosa*	1			
-*Acanthocoris sordidus*	1			
-*Notobitus meleagris*	1			
-*Hygia opaca*	1			
-*Hygia lativentris*	1			
-*Homoeoceus dilatatus*	1			
-*Homoeoceus unipunctatus*	1			
-*Plinachtus bicoloripes*	1			
-*Cletus trigonus*	1			
-*Cletus punctiger*	1			
-*Cletus rusticus*	1			
-*Riptortus linearis*	1			
-*Daclera levana*	1			

[a]Number of sequences obtained for each species. Not all sequences met the requirements outlined in the methods, so fewer sequences were included in analyses than are listed here for some species.

Table S2-2: Sequence information for all *Burkholderiaceae* sequences recovered from broad-headed bugs, root nodules, and soil.

Species	Sample	Loc.	Type	Method	Sequence	OTU	Accession #
A. calcaratus	11BH	CM	abdomen	culturing	11BH379.1	n/a	KF931420
	BHJ35	LIME	crypts	culturing	BHJ35g	n/a	KF931444
	BHB6	SM	crypts	cloning	BHB6c	2	KF931445
	BHJ36	SM	crypts	cloning	BHJ36a	2	KF931446
	BHJ36			cloning	BHJ36e	2	KF931447
	BHJ36			cloning	BHJ36h	2	KF931448
	BHJ36			cloning	BHJ36i	5	KF931449
	C7	CM	abdomen	cloning	C7d	2	KF931450
	C7			cloning	C7e	2	KF931451
	C7			cloning	C7f	2	KF931452
	C7			cloning	C7g	2	KF931453
	C7			cloning	C7i	2	KF931454
	C7			cloning	C7a	3	KF931455
	C7			cloning	C7c	3	KF931456
	C7			cloning	C7j	6	KF931457
	C8	CM	abdomen	cloning	C8d	2	KF931458
	C8			cloning	C8j	2	KF931459
	C8			cloning	C8e	10	KF931460
	FMAC3	FM	crypts	cloning	FMAC3a	1	KF931461
	FMAC3			cloning	FMAC3c	1	KF931462
	FMAC3			cloning	FMAC3e	1	KF931463
	FMAC3			cloning	FMAC3g	1	KF931464
	FMAC3			cloning	FMAC3i	1	KF931465
	FMAC3			cloning	FMAC3j	1	KF931466
	FMAC3			cloning	FMAC3h	7	KF931467
	FMAC3			cloning	FMAC3d	12	KF931468
	FMAC4			cloning	FMAC4a	2	KF931469
	FMAC4			cloning	FMAC4b	2	KF931470
	FMAC4			cloning	FMAC4c	2	KF931471
	FMAC4	FM	crypts	cloning	FMAC4d	2	KF931472
	FMAC4			cloning	FMAC4e	2	KF931473
	FMAC4			cloning	FMAC4f	2	KF931474
	FMAC4			cloning	FMAC4h	2	KF931475
	FMAC4			cloning	FMAC4j	2	KF931476
	FMAC4			cloning	FMAC4g	9	KF931477
	FMAC6	FM	crypts	cloning	FMAC6a	1	KF931478
	FMAC6			cloning	FMAC6b	1	KF931479
	FMAC6			cloning	FMAC6c	1	KF931480

	FMAC6			cloning	FMAC6d	1	KF931481
	FMAC6			cloning	FMAC6f	1	KF931482
	FMAC6			cloning	FMAC6h	1	KF931483
	FMAC6			cloning	FMAC6i	1	KF931484
	FMAC6			cloning	FMAC6e	4	KF931485
	FMAC6			cloning	FMAC6g	4	KF931486
	FMAC6			cloning	FMAC6j	4	KF931487
	FMAC7	FM	crypts	cloning	FMAC7b	1	KF931488
	FMAC7			cloning	FMAC7c	1	KF931489
	FMAC7			cloning	FMAC7e	1	KF931490
	FMAC7			cloning	FMAC7f	1	KF931491
	FMAC7			cloning	FMAC7g	1	KF931492
	FMAC7			cloning	FMAC7h	1	KF931493
	FMAC7			cloning	FMAC7i	1	KF931494
	FMAC7			cloning	FMAC7j	1	KF931495
	FMAC7			cloning	FMAC7d	7	KF931496
	FMAC7			cloning	FMAC7a	13	KF931497
	Joy1	JOY	crypts	cloning	Joy1b	2	KF931498
	Joy1			cloning	Joy1c	2	KF931499
	Joy1			cloning	Joy1d	2	KF931500
	Joy1			cloning	Joy1e	2	KF931501
	Joy1			cloning	Joy1a	2	KF931502
	Joy1			cloning	Joy1g	5	KF931503
	Lime2	LIME	crypts	cloning	Lime2c	2	KF931504
	Lime2			cloning	Lime2e	2	KF931505
	Lime2			cloning	Lime2f	2	KF931506
	Lime2			cloning	Lime2i	2	KF931507
A. conspersus	3BH	SM	crypts	culturing	3bh1624.4	n/a	KF931424
	3BH		abdomen	culturing	3bh3624.1	n/a	KF931425
	BHJ27	MNP	crypts	cloning	BHJ27.2a	2	KF931508
	BHJ27			cloning	BHJ27.2b	17	KF931509
	BHJ27			cloning	BHJ27.2d	17	KF931510
	BHJ27			cloning	BHJ27.2g	17	KF931511
	BHJ27			cloning	BHJ27.2i	17	KF931512
	BHJ27			cloning	BHJ27.2c	29	KF931513
	BHJ27			cloning	BHJ27.2j	29	KF931514
	BHJ28	MNP	crypts	cloning	BHJ28.2f	2	KF931515
	BHJ28			cloning	BHJ28.2h	2	KF931516
	BHJ28			cloning	BHJ28.2d	29	KF931517
	BHJ28			cloning	BHJ28.2i	17	KF931518
	BHJ28			cloning	BHJ28.2a	29	KF931519
	BHJ28			cloning	BHJ28.2j	29	KF931520
	BHJ29	MNP	crypts	cloning	BHJ29.2a	2	KF931521

BHJ29			cloning	BHJ29.2b	2	KF931522
BHJ29			cloning	BHJ29.2d	2	KF931523
BHJ29			cloning	BHJ29.2j	3	KF931524
BHJ29			cloning	BHJ29.2h	2	KF931525
BHJ29			cloning	BHJ29.2c	29	KF931526
BHJ29			cloning	BHJ29.2e	29	KF931527
BHJ30	MNP	crypts	cloning	BHJ30.2e	2	KF931528
BHJ30			cloning	BHJ30.2h	2	KF931529
BHJ30			cloning	BHJ30.2i	2	KF931530
BHJ30			cloning	BHJ30.2j	2	KF931531
BHJ30			cloning	BHJ30.2a	29	KF931532
BHJ30			cloning	BHJ30.2b	30	KF931533
FMAC8	FM	crypts	cloning	FMAC8a	18	KF931534
SM13	SM	abdomen	cloning	SM13d	1	KF931535
SM13			cloning	SM13i	1	KF931536
SM13			cloning	SM13j	4	KF931537
SM2	SM	crypts	cloning	SM2e	1	KF931538
SM2			cloning	SM2a	2	KF931539
SM2			cloning	SM2b	2	KF931540
SM2			cloning	SM2c	12	KF931541
SM2			cloning	SM2f	2	KF931542
SM2			cloning	SM2g	2	KF931543
SM2			cloning	SM2h	2	KF931544
SM8	SM	crypts	cloning	SM8c	1	KF931545
SM8			cloning	SM8d	1	KF931546
SM8			cloning	SM8e	1	KF931547
SM8			cloning	SM8f	1	KF931548
SM8			cloning	SM8g	1	KF931549
SM8			cloning	SM8h	1	KF931550
SM8			cloning	SM8j	1	KF931551
W3	WY	crypts	cloning	W3a	2	KF931552
W4	WY	crypts	cloning	W4a	2	KF931553
W4			cloning	W4c	11	KF931554
W4			cloning	W4d	2	KF931555
W4			cloning	W4e	2	KF931556
W4			cloning	W4f	2	KF931557
W4			cloning	W4g	2	KF931558
W5	WY	crypts	cloning	W5a	2	KF931559
W5			cloning	W5c	2	KF931560
W5			cloning	W5e	2	KF931561
W5			cloning	W5f	2	KF931562
W5			cloning	W5g	2	KF931563
W5			cloning	W5h	2	KF931564

	W5			cloning	W5i	2	KF931565
	W5			cloning	W5j	2	KF931566
	W5			cloning	W5k	2	KF931567
	W5			cloning	W5l	2	KF931568
	W5			cloning	W5m	2	KF931569
A. tomentosus	2BH	SM	abdomen	culturing	2BH3530.7	n/a	KF931423
	2BH			culturing	2BH2530.1	n/a	KF931421
	2BH			culturing	2BH2530.3	n/a	KF931422
	BHB5	SM	abdomen	cloning	BHB5c	2	KF931570
	BHB5			cloning	BHB5g	2	KF931571
	BHB5			cloning	BHB5h	2	KF931572
	BHB7	SM	abdomen	cloning	BHB7i	2	KF931573
	BHB11	SM	abdomen	cloning	BHB11c	2	KF931574
	BHB11			cloning	BHB11e	2	KF931575
	BHJ32	MNP	crypts	culturing	BHJ32b	n/a	KF931437
	BHJ32			culturing	BHJ32c	n/a	KF931438
	BHJ32			culturing	BHJ32d	n/a	KF931439
	BHJ32			culturing	BHJ32g	n/a	KF931440
	BHJ32			culturing	BHJ32i	n/a	KF931441
	BHJ38	SM	crypts	cloning	BHJ38b	2	KF931576
	BHJ38			cloning	BHJ38c	2	KF931577
	BHJ38			cloning	BHJ38d	2	KF931578
	BHJ38			cloning	BHJ38e	2	KF931579
	BHJ38			cloning	BHJ38f	2	KF931580
	BHJ38			cloning	BHJ38g	2	KF931581
	BHJ38			cloning	BHJ38i	2	KF931582
	BHJ38			cloning	BHJ38j	2	KF931583
	BHJ39	SM	crypts	cloning	BHJ39a	2	KF931584
	BHJ39			cloning	BHJ39c	2	KF931585
	BHJ39			cloning	BHJ39d	2	KF931586
	BHJ39			cloning	BHJ39e	2	KF931587
	BHJ39			cloning	BHJ39f	2	KF931588
	BHJ39			cloning	BHJ39g	2	KF931589
	BHJ39			cloning	BHJ39h	2	KF931590
	BHJ39			cloning	BHJ39j	5	KF931591
	BHJ39			cloning	BHJ39i	8	KF931592
	BHJ39			cloning	BHJ39b	9	KF931593
	Elf1	LC	abdomen	cloning	ELF1i	1	KF931594
	Joy2	JOY	crypts	cloning	Joy2j	1	KF931595
	Joy3			cloning	Joy3a	2	KF931596
	Joy3			cloning	Joy3b	2	KF931597
	Joy3			cloning	Joy3c	2	KF931598
	Joy3			cloning	Joy3d	2	KF931599

	Joy3			cloning	Joy3f	2	KF931600
	Joy3			cloning	Joy3g	2	KF931601
	Joy3			cloning	Joy3h	2	KF931602
	Joy3			cloning	Joy3j	2	KF931603
	Joy3			cloning	Joy3e	3	KF931604
	Joy4	JOY	crypts	cloning	Joy4a	2	KF931605
	Joy4			cloning	Joy4b	2	KF931606
	Joy4			cloning	Joy4c	2	KF931607
	Joy4			cloning	Joy4d	2	KF931608
	Lime1	LIME	abdomen	cloning	LIME1a	2	KF931609
	Lime1			cloning	LIME1b	2	KF931610
	Lime1			cloning	LIME1j	2	KF931611
	Lime1			cloning	LIME1c	6	KF931612
	Lime1			cloning	LIME1h	19	KF931613
	Lime4	LIME	crypts	cloning	Lime4b	2	KF931614
	Lime4			cloning	Lime4a	8	KF931615
Megalotomus	BHJ23	LAB	crypts	cloning	BHJ23.2d	1	KF931616
	BHJ23			cloning	BHJ23.2c	8	KF931617
	BHJ31	HM	crypts	culturing	BHJ31b	n/a	KF931426
	BHJ31			culturing	BHJ31c	n/a	KF931427
	BHJ31			culturing	BHJ31d	n/a	KF931428
	BHJ31			culturing	BHJ31f	n/a	KF931429
	BHJ31			culturing	BHJ31h	n/a	KF931430
	BHJ31			culturing	BHJ31k	n/a	KF931431
	BHJ31			culturing	BHJ31l	n/a	KF931432
	BHJ31			culturing	BHJ31n	n/a	KF931433
	BHJ31			culturing	BHJ31o	n/a	KF931434
	BHJ31			culturing	BHJ31p	n/a	KF931435
	BHJ31			culturing	BHJ31q	n/a	KF931436
	BHJ34	CFH	crypts	culturing	BHJ34a	n/a	KF931442
	BHJ34			culturing	BHJ34h	n/a	KF931443
	W2	WY	crypts	cloning	W2a	2	KF931618
	W2			cloning	W2b	2	KF931619
	W2			cloning	W2c	2	KF931620
	W2			cloning	W2e	2	KF931621
	W2			cloning	W2g	2	KF931622
	W2			cloning	W2h	2	KF931623
	W2			cloning	W2j	2	KF931624
	W2			cloning	W2k	2	KF931625
	W2			cloning	W2l	2	KF931626
	W2			cloning	W2d	3	KF931627
L. cuneata	Les1n2	SM	nodule	culturing	Les1n2i	7	KF931405
	SMLes2n1	SM	nodule	culturing	SMLes2n1b	20	KF931406

	SMLes2n2	SM	nodule	culturing	SMLes2n2b	20	KF931407
	SMLes2n2			culturing	SMLes2n2c	20	KF931408
	SMLes2n3	SM	nodule	culturing	SMLes2n3c	23	KF931409
	SMLes2n3			culturing	SMLes2n3b	17	KF931410
	SMLes2n3			culturing	SMLes2n3a	21	KF931411
	SMLes4n2	SM	nodule	culturing	SMLes4n2b	21	KF931412
Soil	SML	SM	soil	culturing	SML1b	20	KF931413
	SML			culturing	SML1d	20	KF931414
	SML			culturing	SML3d	19	KF931415
	SMT	SM	soil	culturing	SMT4a	22	KF931416
	Snap2	SNAP	soil	culturing	Snap2a	1	KF931417
	Snap2			culturing	Snap2c	21	KF931418
	Snap3			culturing	Snap3b	24	KF931419

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Chapter 3

Symbiont winnowing in the squash bug midgut

ABSTRACT

Most host organisms have specific, non-random microbial associates assembled from a variety of sources. Hosts with inherited microbial associates are largely colonized by the same microbial genotypes that were present in their predecessors, but hosts with environmentally acquired microbes are exposed to a large pool of potential symbionts and must have mechanisms in place to establish specific microbial associations. Here I investigate the establishment of specific bacterial associations in the heteropteran squash bug (*Anasa tristis*) by deep sequencing the bacterial inhabitants of the midgut, the route of acquisition to the symbiont-harboring organ (crypts) of the squash bug. Contrary to previous investigations of heteropteran crypts, I found that all squash bugs crypts were inhabited by two bacterial genera, *Burkholderia* and *Wolbachia*, and occasionally *Enterococcus* or Rhizobiales. *Burkholderia* and *Wolbachia* were also the dominant inhabitants in the four midgut regions anterior to the crypts, though their abundance relative to the crypts varied in each region. More than 99% of all of the *Burkholderia* sequences grouped in the same OTU, indicating that squash bugs establish a symbiosis with a very specific *Burkholderia* strain and that this specificity is likely established in the environment or in the host anterior to the midgut. NMDS ordination showed that the bacterial communities in the crypts were very similar and that other midgut regions, host individual, and collection location

were not strong predictors of bacterial community. I discuss the role of bacterial co-infection on the host and suggest further work that should be done to investigate the mechanism for determining bacterial specificity in the squash bug.

INTRODUCTION

It has become increasingly clear that host-associated microbiomes are not a random assemblage of microbes (Song *et al.* 2013; Engel & Moran 2013), nor are they a direct reflection of the host's environment (Webster *et al.* 2012; Fitzpatrick & Allison 2014). Stable and replicable microbial communities have been found in a wide range of host organisms and tissues (Kirk *et al.* 2005; Engel & Moran 2013; Faith *et al.* 2013; Andersen *et al.* 2013). This is especially remarkable in hosts that acquire their microbial associates horizontally from the environment as it suggests host, microbial or abiotic factors -- or some combination of each -- function to select specific microbes out of the huge pool of potential microbial partners to which they are exposed. The factors that determine host-microbe specificity are only starting to be explored (Belda-Baillie *et al.* 2002; Ruby 2008; Visser *et al.* 2009).

There are two important habitats in horizontal microbe acquisition where specificity can be determined: the environment external to the host and within the host. Any ecological factor that increases or decreases a microbe's chance of acquisition before host contact can be considered an environmental screen. Environmental screens typically impact microbial abundance and availability in the host's habitat. Host screening applies to changes in microbial abundance that occur after a host has acquired microbes. Host screens can facilitate colonization of host tissues by certain microbes (screen in) or can block access to certain microbes (screen out). Within these two habitats, the environment and the host, specificity can be determined by both biotic and abiotic components.

The coupled roles of environmental and host screening have been investigated in few host-microbe associations, and many of these involve highly selective and specialized host-symbiont pairings. In the symbiosis between bobtail squid (*Euprymna scolopes*) and the environmentally-acquired bacterium *Vibrio fischeri,* for example, host screening appears to play a larger role than environmental screening. In the environment there can be a slightly higher concentration of *Vibrio fischeri* in localized squid habitats (Lee & Ruby 1994), but overall *V. fischeri* are rare relative to other bacterioplankton, making up as little as 0.1% of the population (Nyholm & McFall-Ngai 2004). Host screening, on the other hand, is highly selective. It is both a “screen-in” and "screen-out" process, wherein *V. fischeri* but not most other bacterial cells aggregate in mucus produced on the squid’s mantle and then migrate through a duct with high concentrations of reactive oxygen species that are not tolerated by non-*V. fischeri* cells (Nyholm & McFall-Ngai 2004). In contrast, the symbiosis between leguminous plants and rhizobial bacteria is largely a screen-in process where both partners must produce a series of molecules call *nod* factors to signal that they are appropriate partners. This signaling induces the growth of the plants' symbiont-housing root nodules (Janczarek *et al.* 2015).

Like squid and leguminous plants, many true bug (Heteropteran) insects also acquire specific symbiotic bacteria from the environment that are stored within specialized regions of the hosts' guts, called crypts (Kikuchi *et al.* 2007; 2012). These bacteria are acquired during a limited developmental window in the second nymphal stage, after which the host becomes largely refractory to acquiring

symbionts from the environment (Kikuchi *et al.* 2011a), and can reach densities of ten million bacteria per bug (Kikuchi & Fukatsu 2014). Previous research indicates these crypts tend to harbor a narrow subset of bacteria (Olivier-Espejel *et al.* 2011; Kikuchi *et al.* 2011b; Boucias *et al.* 2012; Garcia *et al.* 2014), mostly in the *Burkholderia* genus, of the diverse assemblage of bacteria in the environment that are likely ingested during feeding and probing (Kikuchi *et al.* 2007; Itoh *et al.* 2014). In some heteropteran species, *Burkholderia* in the crypts are likely a monoculture within each individual, with identical or very similar *Burkholderia* present in all individuals of the same species (Kikuchi *et al.* 2005; 2007). In other host species, three to seven *Burkholderia* strains have been found across populations of the same hosts species (Olivier-Espejel *et al.* 2011; Boucias *et al.* 2012; Itoh *et al.* 2014) (Kikuchi *et al.* 2011b), with co-infections occurring occasionally (Kikuchi *et al.* 2011b; Itoh *et al.* 2014). In totality, the relationship between heteropteran hosts and *Burkholderia* has been described as "ancient but promiscuous", indicating *Burkholderia* have been hosted by heteropterans over a long evolutionary time, but there have been horizontal transfers of symbionts from the environment and other hosts and no strict host-symbiont co-evolution has occurred (Kikuchi *et al.* 2011b).

Host screening seems to play a significant role in determining the specificity of these relationships. As Heteropteran crypts are modified regions of the posterior midgut, an organ that is sequestered from environmental contact, earlier points on the acquisition route including the mouthparts, the foregut, and the frontal portion of the midgut may serve as the site of host screening, or screening may be concentrated near or within the actual symbiont crypt. The m4b region, the organ

that leads directly into the crypts (see Fig.3-1), has been shown to have antimicrobial activity that acts specifically against *Burkholderia* in the crypts, though this may function more in regulating the symbiont population in the crypts after it is well-established (Kim *et al.* 2013b). However this antimicrobial activity is stronger in insects that have not yet been colonized by *Burkholderia*, suggesting it may act to impose or maintain specificity during acquisition, though it is not known whether this activity is effective against non-established *Burkholderia* or other bacteria (Kim *et al.* 2014). There is also evidence that *Burkholderia* with specific characteristics are "screened in" and allowed access to the crypts during acquisition. *Burkholderia* deficient in a cell wall synthesis gene, a purine biosynthesis gene, or flagellar production all fail to colonize and establish in the crypts at different points during acquisition (Ohbayashi *et al.* 2012; Kim *et al.* 2013c; a).

The structure of the Heteropteran midguts, with clear sectioning and a specific region dedicated to housing specific symbionts (Kikuchi *et al.* 2011b), provides an opportunity to investigate the process of within-host winnowing of the bacterial community along an acquisition route. Here, I use Illumina MiSeq to sequence five sections of *Anasa tristis* (the squash bug) from multiple individuals and describe the bacterial communities. Previous research indicates that in this species, similar to many others Heteropterans, the midgut crypts are dominated by bacteria in the genus *Burkholderia* (Acevedo et al, in prep). It is unknown whether other bacteria coexist in the crypts. Given that this concentration of *Burkholderia* is not representative of the environment (Garcia *et al.* 2014), host screening likely dictates this specificity, though it is unclear whether that screening occurs within

the larger midgut or within the crypt itself. To investigate this, I compare the prevalence of *Burkholderia* within the microbial communities in different sections of *A. tristis* midguts. I then focus specifically on the crypts to determine i) whether *Burkholderia* are truly the only bacterial inhabitants, and ii) whether the crypt population is a monoculture or polyculture of *Burkholderia* spp.

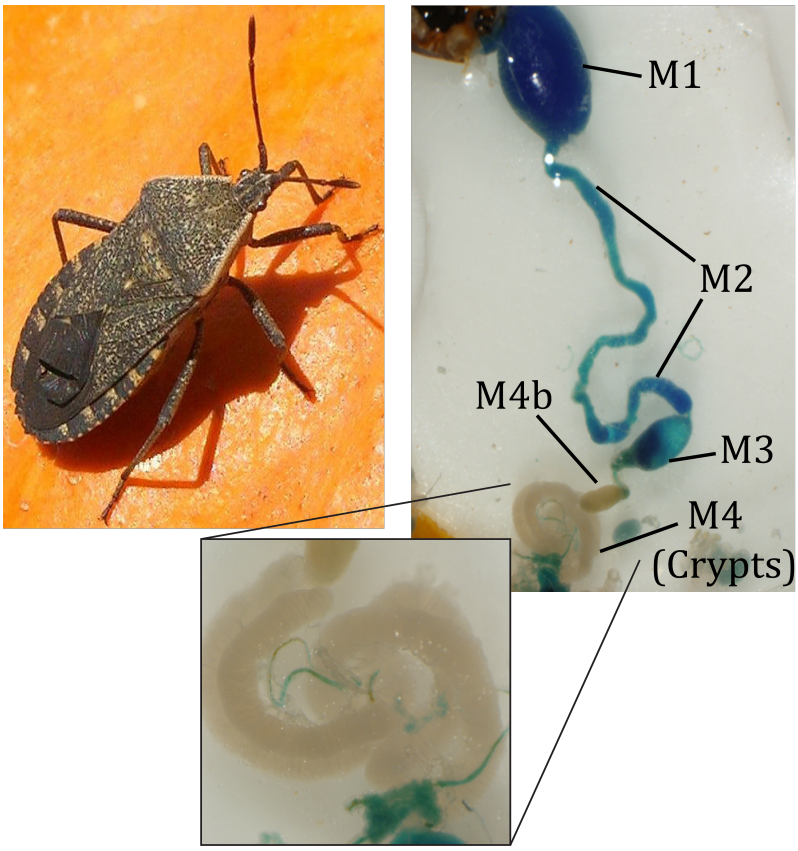

Figure 3-1. The squash bug, *Anasa tristis*, and the structure of its midgut with the five distinct regions labeled.

MATERIALS AND METHODS

Squash Bug Collection and Dissection

Adult squash bugs (*Anasa tristis*) were collected from four locations: two organic farms in Northern Georgia (n = 5 from each location), one community garden in suburban Atlanta, Georgia (n = 5), and a garden in Columbia, Missouri (n = 5). Dissections were performed as soon as possible after collection. When squash bugs were dissected more than a few hours after collection, they were maintained in the lab on leaves and fruit from the collection site and were housed only with squash bugs collected from the same site. Squash bugs were anesthetized with CO_2 gas and then sacrificed and surface-sterilized in 95% ethanol for 5 minutes. Each bug was rinsed in sterile Carlson's solution, a buffer used to keep insect and bacterial cells viable but non-proliferative (Mitsuhashi 2002), and then the insect was pinned to a dissection dish and covered with fresh Carlson's solution. The lower ventral cuticle of each bug was removed by cutting around each side of the abdomen with sterilized micro-scissors, and organs other than the midgut were removed. The connective tissue that keeps the midgut coiled was then clipped to straighten the midgut, and each organ was removed starting by clipping the crypts from hindgut. The midgut regions taken from each bug that were used for sequencing are presented in Table 1; in total, sequencing was conducted on 20 crypts samples, six m1 region samples, five m2 region samples and m3 regions samples, and four m4b region samples (see Fig. 3-1). Each organ was rinsed with Carlson's solution and then placed in 200 μL of fresh Carlson's solution and homogenized with a sterile

pestle. Tissue samples were stored at -20° C until further processing.

DNA extraction, PCR amplification, and Sequencing

A phenol-chloroform method was used to extract and purify gDNA from each tissue sample. An equal volume of cetyl trimethylammonium bromide (CTAB) was

Table 3-1: Bug samples, their collection locations, and the midgut regions sampled.

		Midgut Region				
Bug	**Location**	M1	M2	M3	M4b	M4
SB12	Woodland			X		X
SB13	Woodland					X
SB14	Woodland			X		X
SB15	Woodland		X			X
SB16	Oakhurst		X			X
SB17	Oakhurst	X				X
SB18	Oakhurst	X				X
SB19	Woodland				X	X
SB20	Oakhurst	X				X
SB21	Oakhurst	X	X			X
SB22	Crystal					X
SB24	Crystal			X		X
SB25	Crystal			X		X
SB26	Crystal	X				X
SB27	Crystal		X	X		X
SB28	Missouri				X	X
SB29	Missouri				X	X
SB30	Missouri				X	X
SB31	Missouri	X	X			X
SB32	Missouri					X

added to each sample and then incubated at 60° C for one hour. Sodium dodecylsulfate (SDS) was added to a final concentration of 2% and each sample was incubated again at 60° C for one hour. Nucleic acids were extracted with an equal volume of 24:24:1 phenol:chloroform:isoamyl alcohol and then extracted twice with an equal volume of chloroform. Two volumes of cold 99.5% ethanol and 0.1 volumes of NaOAc were added, and then samples were placed at -20° C to precipitate overnight. Pelleted DNA was washed with 75% ethanol, dried, and re-suspended in molecular grade water. The V4 region of the 16S rRNA gene was amplified using dual-indexed primers (Kozich *et al.* 2013) that created a unique nucleotide tag for each sample. The 5 PRIME PCR kit was used to amplify the V4 region of the 16SrRNA gene in 25 uL reactions containing. Reactions were denatured at 94° C for 2 mins followed by 30 cycles at 94° C for 20 s, either 55° C or 60° C for 15 s, and 72° C for 5 min with a final elongation step at 72° C for 10 min (modified from (Kozich *et al.* 2013). Amplicons were purified with the Qiagen PCR Purification kit, eluted with molecular grade water, and quantified with the Qubit dsDNA BR Assay kit using the Qubit Fluorometer. Samples were pooled in equimolar concentrations and then run on the Illumina Miseq with the MiSeq Reagent Kit v2.

Bioinformatic Analysis

MiSeq data was analyzed following a standard pipeline (Kozich *et al.* 2013) in mothur (Schloss *et al.* 2009). The paired end reads were assembled into contigs and screened to remove contigs that were the wrong length, had ambiguous reads,

or had a high number of hompolymers (> 8). The remaining contigs were dereplicated and then aligned to template sequences from the SILVA database. All unnecessary gap characters were removed from the alignment, and chimeras and other erroneous sequences were removed. Contigs were classified using a naïve Bayesian classifier, and sequences that were identified as archaea, eukaryotes, mitochondria, or cholorplasts were removed. Contigs were clustered into OTUs at 0.03 distance using the cluster.split method and a representative contig from each OTU was classified using the Bayesian classifier.

Data Analysis

The 50 most abundant OTUs were ordinated to visualize differences based midgut region, geographic location, and individual bug using the phyloseq package {McMurdie:2013dm} in R version 3.1.3. The Bray-Curtis dissimilarity index was used to perform nonmetric multidimensional scaling (NMDS) on a sample x OTU matrix that included abundance data. Permutational MANOVA (PERMANOVA) was used to test for significant differences in the community structure of the 50 most abundant OTUs among midgut regions and collection locations with the adonis function in the vegan package {veganCommunityEco:2013uw}. Only m4 midgut regions were included in the test for differences among collection locations as there was not an even representation of the other midgut regions across collection locations. PERMANOVA was run on a Bray-Curtis dissimilarity matrix with 10,000 permutations.

RESULTS

Overall bacterial midgut community

We formed 6,541,731 paired end 16S V4 rRNA gene contigs with lengths between 250 and 275 base pairs from 40 midgut samples. These sequences grouped into 290 genera within 144 families. The 50 most abundant OTUs, which accounted for more than 98% of the sequences, contained only five genera including *Burkholderia*, *Lautropia* (a sister genus to *Burkholderia*), *Wolbachia*, *Enterococcus*, and a group of strains not classified to genus; these were from the Enterobacteriaceae, Rhizobiales, Pseudomonadaceae, Bacillales, Myxococcales, and Rickettsiales orders (Fig. 3-2). *Wolbachia* was the most abundant genera in the m1, m2, and m4b regions and *Burkholderia* was the most abundant genera in the m3 and m4 regions. The 20 squash bug crypts we sampled contained a mix of *Burkholderia*, *Wolbachia*, and unclassified species in the Rhizobiales, though *Burkholderia* were numerically dominant in all of the crypts samples.

Bacterial communities across individual bugs, midgut regions, and locations

I used PERMANOVA and NMDS plots to evaluate differences in bacterial community composition (including abundances) by midgut region and collection location. There was a significant difference among midgut regions (PERMANOVA, $F = 2.991$, d.f. = 4, $p = 0.0001$) and in an NMDS plot crypt samples (m4) formed a cohesive group that was largely separate from the other midgut regions (Fig. 3-3A).

The remaining midgut samples did not group by gut region. Crypt samples collected at the same location did not form distinct groups in the NMDS plot (Fig. 3-3B), but were shown to be significantly different with a PERMANOVA test (PERMANOVA, F = 2.4191, d.f. = 3, p = 0.0312). There was no distinct grouping of samples by individual bug in the NMDS plot (this ordination only included samples from bugs from which the m4 and another midgut region were sequenced; Fig. 3-4).

Based on 16S rRNA, Burkholderia *is a monoculture throughout the midgut*

Burkholderia were present in every organ we sampled except one m3 sample, indicating presence throughout the midgut. All sequences that were classified in the *Burkholderia* genus with a 90% or greater probability were clustered into OTUs separately from the whole bacteria dataset. The 2,094,230 sequences identified as *Burkholderia* grouped into 11 OTUs, one of which was highly dominant (99.999% of all *Burkholderia* reads).

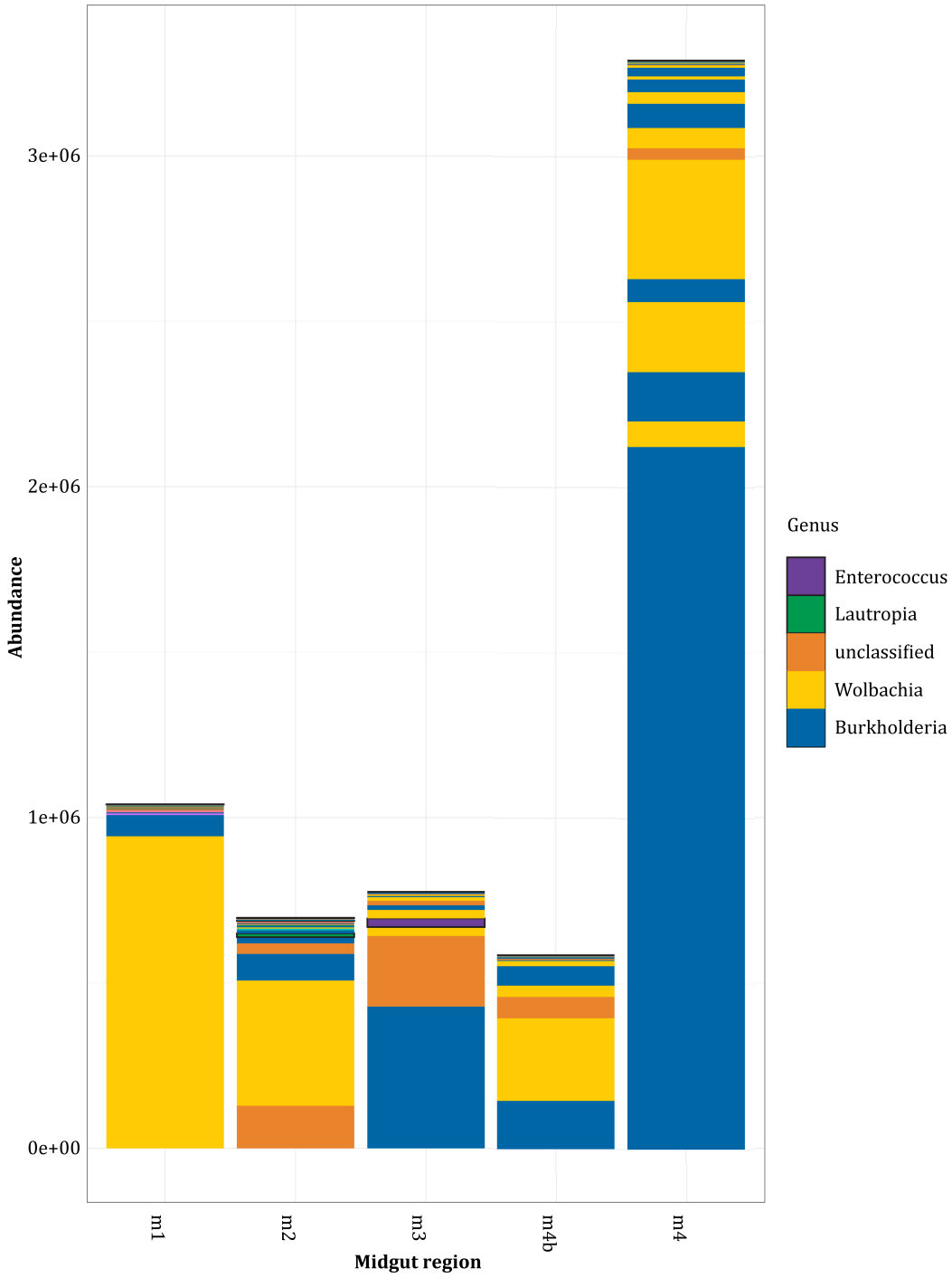

Figure 3-2. Absolute abundance of paired end reads for the 50 most abundant OTUs classified by genera.

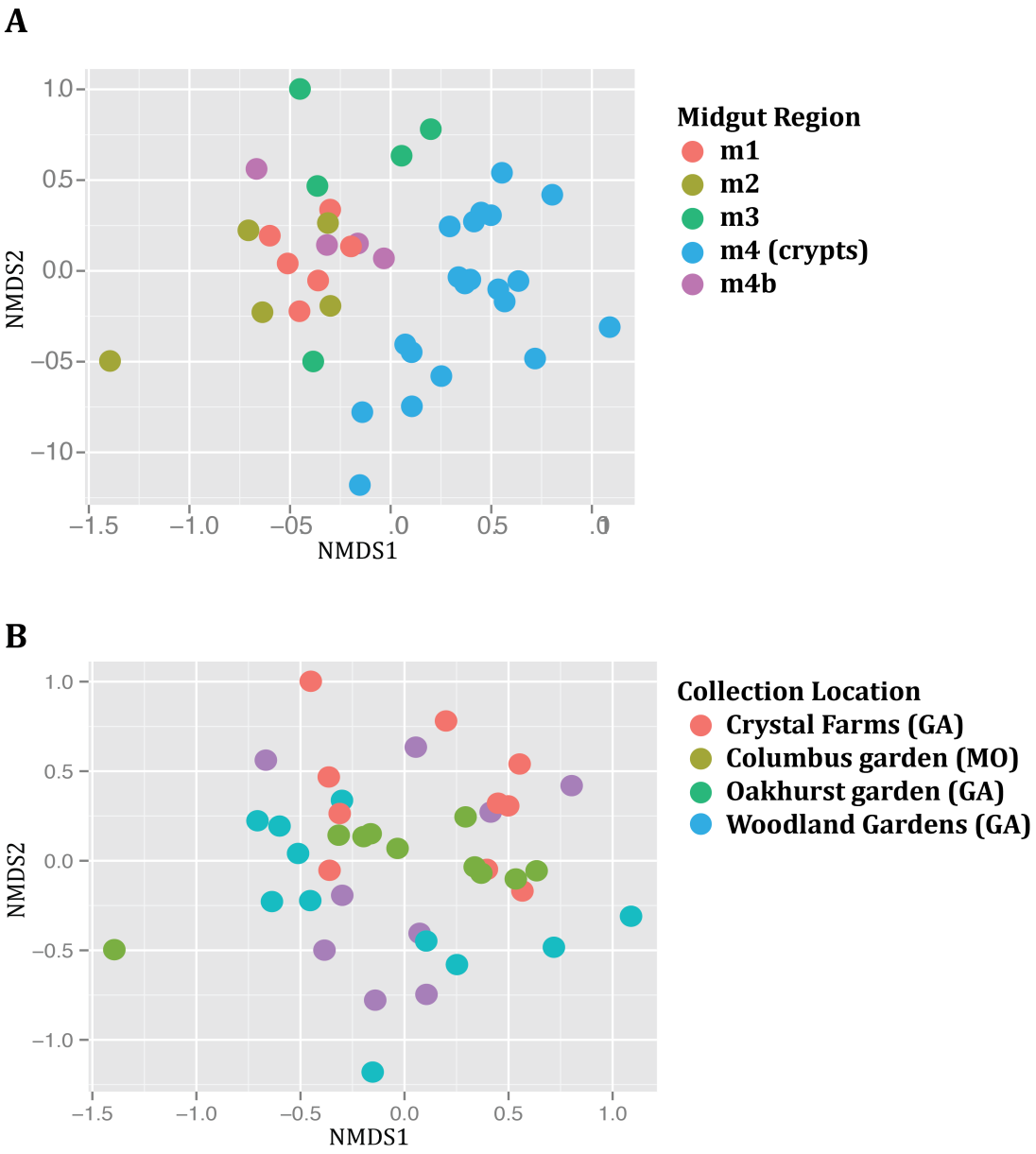

Figure 3-3. NMDS plots of the total bacterial community of each sample represented by a plot point coded by A) midgut region or B) geographic collection location. Crypts are called m4 here.

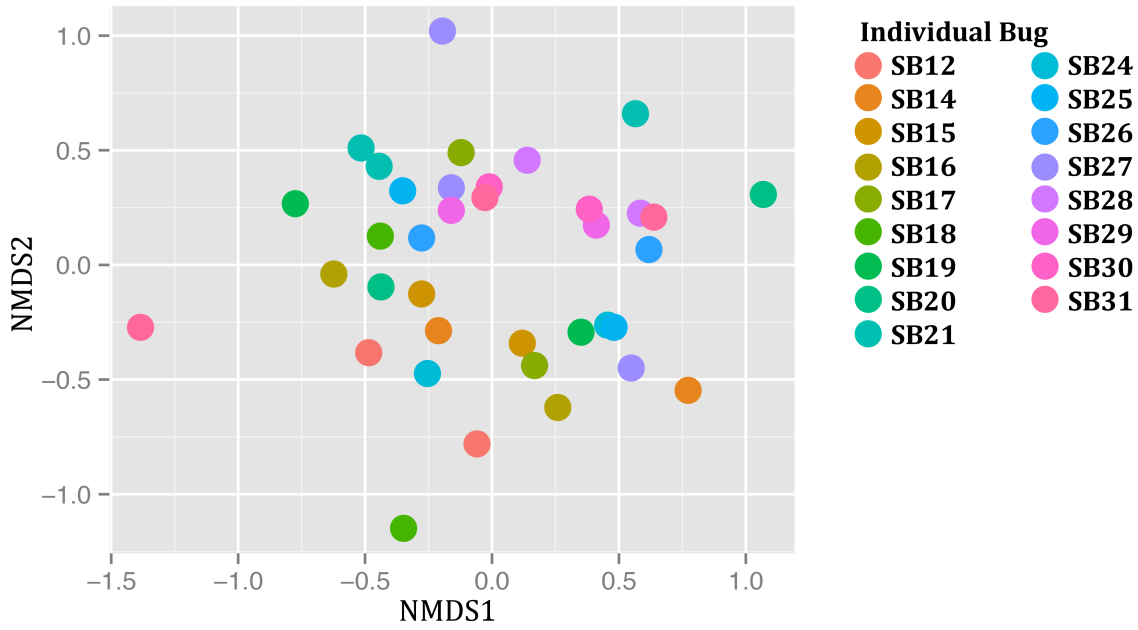

Figure 3-4. NMDS plot of the total bacterial community of each sample represented by a plot point coded by individual bug. Only midgut sections from bugs that contained two or more samples are included in this plot.

DISCUSSION

The squash bug midgut is dominated by a monoculture of *Burkholderia*, though a number of other bacteria, such as *Wolbachia*, are present in each midgut region, including the symbiont-specialized midgut crypts. Previous investigations of other heteropterans, including coreids, have indicated that *Burkholderia* are likely the only bacterial inhabitants of the midgut crypts (Kikuchi *et al.* 2011b; Boucias *et al.* 2012; Garcia *et al.* 2014). However, those studies have relied on a combination of culturing, which would not detect *Wolbachia*, and small-scale sequencing, which may have been insufficient to detect less abundant bacteria. Illumina deep

sequencing done on the crypts of another heteropteran, the oriental chinch bug, did not show that the crypts contained any bacteria other than *Burkholderia*(Itoh *et al.* 2014), though *Wolbachia* was recovered from the giant mesquite bug using Sanger sequencing (Olivier-Espejel *et al.* 2011). It is unknown if the presence of Rhizobiales and *Wolbachia* in the crypts is specific to the squash bug or a more widespread pattern in other heteropterans.

Coinfection of hosts with multiple bacterial symbionts has been increasingly detected in molecular surveys. In contrast to coinfection with pathogens, which has a great deal of theoretical and evidence-based prediction of outcome for hosts (de Roode *et al.* 2005; Vasco *et al.* 2007; Sternberg *et al.* 2011), there has been little investigation of the effect of symbiont (used here to indicate a beneficial relationship with a host) coinfection on hosts or the symbionts. However, coinfection can be an important mediator of symbiont and host phenotypes. Hosts can benefit from co-infection as some symbiont combinations can produce additive or multiplicative effects on host fitness(Oliver *et al.* 2006). For example, a leguminous shrub experiences a synergistic increase in biomass production when coinfected with rhizobial bacteria and mycorrhizal fungi (Larimer *et al.* 2014). Coinfections can also have neutral or negative effects on the host (Mouton *et al.* 2004; Oliver *et al.* 2006; White *et al.* 2009; 2010). Similarly, coinfection can augment or suppress the density of symbionts in host tissues (Oliver *et al.* 2006), an effect which is particularly prevalent when at least one of the coinfecting strains is *Wolbachia* (Mouton *et al.* 2004; Goto *et al.* 2006). Since *Wolbachia* is likely present in the midgut before *Burkholderia*, as *Wolbachia* is maternally transmitted, it is

possible that it plays a role in the specificity of the host-*Burkholderia* association.

Although other bacteria were in the squash bug crypts, only one strain of *Burkholderia,* based on 16S rRNA genotype, was exceedingly dominant. Both clonal and heterogeneous *Burkholderia* populations have previously been reported in heteropteran crypts (Kikuchi *et al.* 2005; 2011b; Itoh *et al.* 2014; Garcia *et al.* 2014). There are three distinct *Burkholderia* strains that colonize the crypts of the oriental chinch bug, but there is typically a monoculture of one strain within each bug and double or triple coinfections are rare (Itoh *et al.* 2014). This seems to be a somewhat widespread pattern wherein there are two or three *Burkholderia* strains that are preferred in many heteropterans, but there seems to be only one dominant strain within each host (Kikuchi *et al.* 2005; Itoh *et al.* 2014). In the squash bug, though, there seems to be only a single preferred strain that is present in all hosts regardless of collection location. Further work, using quantitative methods in other systems is needed to confirm whether this is found in other insect systems as well. Work is also needed to explore the importance of genetic variation throughout the symbiont genome. It is known, for example, that strains of *Burkholderia* associated with another heteropteran, the bean bug *Riptortus pedestris*, can vary in their capacity to confer pesticide resistance to their insect hosts (Kikuchi *et al.* 2012). In this case, they are identical at the 16S rRNA locus but vary at loci important for the symbiosis.

In addition to the crypts, this strain of was the only dominant *Burkholderia* OTU present throughout the midgut. This indicates that symbiont screening likely occurs before bacteria colonize the host midgut, but it is unknown whether it is mostly due to environmental or host screening. The acquisition point for bacteria is

the rostrum, which, in addition to other mouthparts and the forgeut, is anterior to the midgut and could be the site of host filters for this specific strain of *Burkholderia* through the production of a bactericidal compound or other mechanism. However, screening could also occur before host contact. In a previous study, symbiotic *Burkholderia* strains were depleted in the soil compared to their population within a host, one of four species of broad-headed bug (Garcia *et al.* 2014), and so it seems unlikely that the chances of this *Burkholderia* strain being acquired would be boosted by an increased abundance in the soil. However, the environmental source of the squash bug symbionts is not known, and environmental screening could occur by preferential growth of the *Burkholderia* strain on the squash bugs' food plants or by some mechanism that makes this strain easier to acquire, such as adhesion factors.

Ten other *Burkholderia* OTUs were detected, which accounted for a combined 0.001% of the *Burkholderia* sequences recovered. As most of these OTUs were recovered from the crypts of a single bug at very low frequencies (1 – 15 sequences), they may be mutations arising in the clonal population that are likely not persistent members of the community.

It is unsurprising that *Wolbachia* was detected in the squash bug, as over 60% of insects, including heteropterans (Kikuchi & Fukatsu 2003), harbor or are predicted to harbor *Wolbachia* infections (Hilgenboecker *et al.* 2008). *Wolbachia*, intracellular symbionts that typically cause various reproduction manipulation phenotypes including male-killing and cytoplasmic incompatibility, are typically

found in reproductive tissues but have been detected in the midgut and other somatic tissues in *Drosophila* (Goto *et al.* 2006; Osborne *et al.* 2012), mosquitoes (Zouache *et al.* 2009), tsetse flies (Cheng *et al.* 2000), ants (Andersen *et al.* 2012; Frost *et al.* 2014), and other insects (Dobson *et al.* 1999). The *Wolbachia* present in these somatic tissues, especially in the midgut, likely have effects on the host other than on reproduction. In bedbugs, where *Wolbachia* is concentrated in bacteriomes outside reproductive tissues, *Wolbachia* is a nutritional symbiont that likely provides B vitamins to its host (Hosokawa *et al.* 2010; Nikoh *et al.* 2014). A similar relationship has been predicted in leafcutter ants, where *Wolbachia* exist in the gut as extracellular symbionts (Andersen *et al.* 2012). *Wolbachia* in non-reproductive tissues in other insects mediates host protection or vectoring of viruses and other pathogens (Moreira *et al.* 2009; Osborne *et al.* 2012). Given its widespread abundance throughout the midgut, *Wolbachia* likely has an effect on the squash bug host though functional assays and further investigation of its tissue distribution are required to determine its significance.

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Chapter 4

The symbiont side of symbiosis: do microbes really benefit?

Modified from Garcia and Gerardo, NM. 2014. The symbiont side of symbiosis: do microbes really benefit? *Frontiers in Microbiology* 5: 510 - 515.

ABSTRACT

Microbial associations are integral to all eukaryotes. Mutualism, the interaction of two species for the benefit of both, is an important aspect of microbial associations, with evidence that multicellular organisms in particular benefit from microbes. However, the microbe's perspective has largely been ignored, and it is unknown whether most microbial symbionts benefit from their associations with hosts. It has been presumed that microbial symbionts receive host-derived nutrients or a competition-free environment with reduced predation, but there have been few empirical tests, or even critical assessments, of these assumptions. I evaluate these hypotheses based on available evidence, which indicate reduced competition and predation are not universal benefits for symbionts. Some symbionts do receive nutrients from their host, but this has not always been linked to a corresponding increase in symbiont fitness. I recommend experiments to test symbiont fitness using current experimental systems of symbiosis and detail considerations for other systems. Incorporating symbiont fitness into symbiosis research will provide insight into the evolution of mutualistic interactions and cooperation in general.

INTRODUCTION

Microbes have been recognized as an important force in eukaryotic evolution (McFall-Ngai *et al.* 2013), but recognition of the impact of eukaryotes on microbial evolution has lagged behind. Interspecies interactions between microbes and eukaryotic hosts fall on a continuum from parasitism to mutualism. Fitness effects of these interactions are routinely investigated in hosts, but it is necessary to consider both partners to understand how interactions evolve and persist. There is a robust framework for understanding how parasitic interactions promote the fitness of parasitic microbes (pathogens), but the microbe's perspective has largely been ignored in putatively mutualistic interactions, and it is unknown whether most non-parasitic microbes benefit from host association.

Most research of mutualisms has focused on the host, as they are larger and usually a more tractable experimental organism. The effect of microbial association on hosts is routinely tested by comparing fitness in hosts with and without symbionts [Fig. 4-1A; (Kikuchi *et al.* 2007)]. Analogous experiments for symbionts are rarely performed, even in well-described systems. It is often assumed that symbiont fitness is higher in hosts relative to other niches because they receive a competition-free environment, reduced predation, or host-derived nutrients. Population size is a straightforward way to measure microbial fitness (*i.e.,* the replication capacity of a clonal population), but it should be used to quantify symbiont fitness in the same way that it is for hosts - as the difference in replication in the presence and absence of its interacting partner. When tested, some experiments have shown that symbionts suffer deleterious effects or costs, such as

suppressed growth in hosts (Ahmadjian 1993; Kikuchi *et al.* 2007; Wooldridge 2010; Login *et al.* 2011; McFall-Ngai *et al.* 2013; Udvardi & Poole 2013). The presence of some costs in the host relative to other niches does not necessarily preclude the symbiont from gaining a net fitness benefit through host association [*e.g.*, acquiring genetic diversity through horizontal gene transfer (HGT)], but it does suggest an important aspect that should be considered.

The semantics of symbiosis may be partially to blame for the neglect of microbes. There have been two prominent uses of "symbiosis" over the past century. The first follows from the definition of symbiosis by de Bary as "the living together of unlike organisms" and is applied to interspecies associations regardless of the relationship [parasitism, commensalism, or mutualism; (Douglas 2010; Leigh 2010). In the second, symbiosis is synonymous with mutualism and indicates a generally beneficial relationship. This is usually applied when it is known that the host benefits from an association and implies that the symbiont does as well. Here we consider any long-term, intimate association to be a "symbiosis" while reserving mutualism for only those interactions known to be beneficial for both partners.

Here I evaluate evidence for reciprocal benefit in presumed mutualistic microbial symbioses, emphasizing environmentally acquired (horizontal) microbial symbionts in eukaryotic hosts. I also re-examine the role of hosts and microbes in symbioses in light of evidence for symbiont benefit. Although it has previously been recognized that symbionts must be more thoroughly investigated (Bronstein 2001; Wilkinson & Sherratt 2001), recent advances in technology and new study systems

provide novel tools and opportunities for investigating the symbiont side of symbiosis.

AN EVALUATION OF ASSUMED SYMBIONT BENEFITS

Competition

It is assumed that microbial symbionts benefit from a competition--free environment inside hosts because they live in the absence of other microbes that compete for resources. While some systems have monoclonal symbiont populations (Gage 2002; Martens *et al.* 2003; Kubota *et al.* 2007; Dubilier *et al.* 2008; Aanen *et al.* 2009), likely due to bottlenecks during repeated vertically transmission or winnowing during horizontal transmission, not all host-symbiont associations are monoclonal. Within-host competition between strains is important for pathogen fitness (Bell *et al.* 2006) and some vertical symbionts (Oliver *et al.* 2006). This is likely also true for horizontal symbionts as hosts from many systems harbor multiple symbiont genotypes (Baker & Romanski 2007; Dubilier *et al.* 2008; Fay *et al.* 2009; Van Horn *et al.* 2012; FitzPatrick *et al.* 2012; Garcia *et al.* 2014). Even hosts with strict colonization requirements and entry mechanisms, like bobtail squid which select specific strains of *Vibrio fischeri* from diverse microbes in seawater, contain multiple symbiont genotypes (Wollenberg & Ruby 2009).

Competition in a polyclonal symbiont population can result in decreased growth for one species or genotype (Elliott *et al.* 2009; Baker *et al.* 2013; Engelmoer *et al.* 2014) or lower symbiont titers (Mouton *et al.* 2004). Mycorrhizal fungi, for instance, have lower abundance in plant roots when co-inoculated relative to single

inoculations. Furthermore, competition between these fungi is stronger within the host compared to the rhizosphere (Engelmoer *et al.* 2014). Coexistence with other symbionts, however, can be beneficial. Double or triple infections of *Wolbachia* in the wasp *Asobara tabida*, for example, increase the abundance of a specific *Wolbachia* genotype relative to single infections with that genotype only (Mouton *et al.* 2004). Co-infections, therefore, are a necessary but not sufficient condition for competition and there is no a general framework for predicting the conditions in which co-infections will promote or hinder a symbiont's fitness. Future research on within-host competition is needed, and should be considered in the context of mechanisms, such as partner choice and sanctioning, that may reduce or prevent polyclonal infections and competition (Bull & Rice 1991).

Predation and the host immune system

In non-host environments, microbes are attacked by pathogens and preyed upon by predators such as nematodes, zooplankton, and filter-feeding invertebrates. In hosts, symbionts still face pressures akin to predation. Hosts have potent immune defenses with which both horizontal (Dunn & Weis 2009) and vertical (Wang *et al.* 2009; Laughton *et al.* 2011) symbionts must sometimes contend. These defenses are analogous to predators as they suppress population growth and can eliminate organisms from an environment (Sachs & Wilcox 2006; Kim *et al.* 2013). In some cases, a multitude of bacteria enter a host but cannot pass increasingly specific checkpoints to establish within the host (Nyholm & McFall-Ngai 2004; Kim *et al.* 2013). Microbes are killed by a range of host immune responses, including

phagocytosis, antimicrobial peptides, and reactive oxygen species (Davidson *et al.* 2004; Login & Heddi 2013). Hosts can also suppress or regulate established symbiont populations. Carpenter ants reduce bacterial symbiont populations through modulation of an immune response during development (Ratzka *et al.* 2013). Similarly, tsetse flies express antimicrobial peptides in symbiont-housing cells to regulate symbiont populations (Login *et al.* 2011). Although it is not known if host control of symbiont growth via immune system 'predation' is universal, it is clear that symbionts do not grow unfettered in hosts.

Symbiont growth may also be controlled using mechanisms unconnected to the immune system. Rhizobia root nodule bacteria (Udvardi & Poole 2013), algal symbionts of corals (Wooldridge 2010), insect bacterial symbionts (Login & Heddi 2013), and lichen photobionts (Ahmadjian 1993) can have lower growth rates relative to their free-living counterparts. The growth of *Symbiodinium* algae is suppressed in corals relative to free-living *Symbiodinium*, but the rate of photosynthesis is comparable in both populations (Muscatine *et al.* 1984; Falkowski *et al.* 1993), suggesting algal energy is directed towards producing photosynthate for the host rather than self-growth. In other hosts, proliferating *Symbiodinium* cells are preferentially expelled over non-proliferating cells (Baghdasarian & Muscatine 2000). However, growth suppression of certain symbiont cells in the host does not single-handedly indicate a deleterious effect on symbionts. The real indicator of a beneficial association is an increased capacity to reproduce in the host relative to the non-host niche, which has not been sufficiently addressed.

Host-provided nutrition

There are clear examples in which symbionts receive nutrients like amino acids (Graf & Ruby 1998; Macdonald *et al.* 2012) from hosts. Rhizobia bacteria receive numerous compounds from their plant hosts, including amino acids, sugars, and trace ions (Prell *et al.* 2009; Udvardi & Poole 2013). However, it is unclear whether any of these nutrients are beneficial to the symbiont. In the case of amino acids, free-living and cultured rhizobia can synthesize branched chain amino acids on their own, but the synthesis of these amino acids is significantly down-regulated in root nodules, and rhizobia in the host rely solely on the plant for these amino acids (Prell *et al.* 2009). In this state, 'symbiotic auxotrophy', bacteria seem to function more as ammonia-producing organelles rather than organisms seeking to increase their fitness. Similarly, *V. fischeri*, bobtail squid symbionts, receive amino acids, fatty acids and chitin from their hosts (Graf & Ruby 1998; Gage 2002; Martens *et al.* 2003; Jones & Nishiguchi 2006; Kubota *et al.* 2007; Dubilier *et al.* 2008; Aanen *et al.* 2009; Wier *et al.* 2010). However, there is evidence that *V. fischeri* benefit from these host-derived nutrients or another aspect of host association, as environmental populations are larger in habitats with squid hosts compared to those without squid (Lee & Ruby 1994; Bell *et al.* 2006; Jones *et al.* 2007). Ultimately measures of microbial growth along with direct tests of the fate of microbes inside and outside hosts are crucial for understanding the effect of host-derived nutrients.

RECOMMENDATIONS FOR INVESTIGATING SYMBIONT FITNESS

The effect of microbes on hosts has been quantified in many systems by measuring fitness in symbiotic and aposymbiotic hosts, but the effect of host-

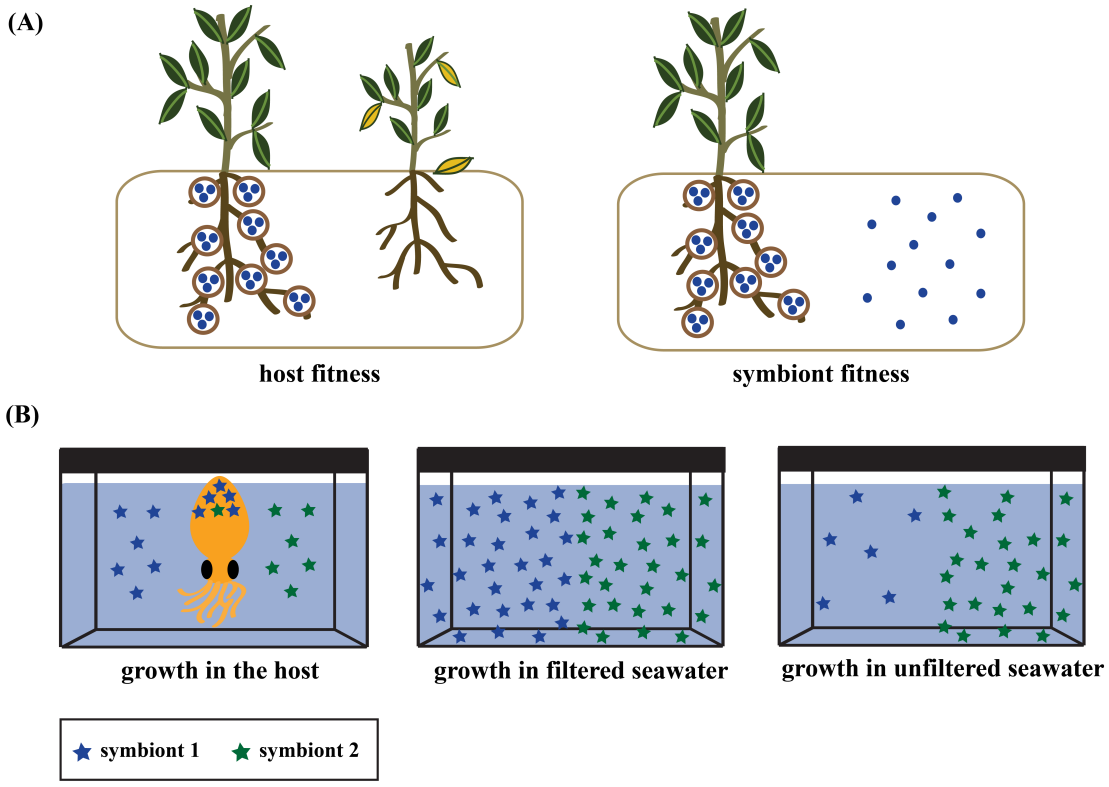

Figure 4-1. A) Experimental designs to test the effect of symbiosis on host fitness (left) and symbiont fitness (right). Both experiments involve measuring growth or other fitness parameters (see recommendations section) in the presence and absence of their partner. Experiments on host fitness have been performed in diverse systems, but the equivalent symbiont fitness experiment is rarely performed. **B)** Experimental design from Wollenberg and Ruby (2012) for measuring the relative growth of two groups of bobtail squid symbionts within naturally infected hosts. Competition assays were performed to test within-host fitness by inoculating the seawater of a hatchling squid with a symbiont strain from each symbiont group (left). A separate experiment confirmed that the symbionts had an equal ability to colonize the squid after single-strain inoculations (not pictured). Symbiont growth was tested in the environment by inoculating filtered (middle) and unfiltered (right) seawater from the natural habitat of the squid and symbiont.

association on symbionts has been tested far less frequently (Fig. 4-1A). One experiment, in the squid-*Vibrio* system, serves as a model for symbiont experiments

using the comparative fitness approach (Fig. 4-1B). Wollenberg and Ruby (2012) inoculated bobtail squid, filtered seawater, and unfiltered seawater with *V. fischeri* strains that were either highly prevalent or rare symbionts in squid hosts. The common symbionts grew as well as the rare symbionts in the squid host and in filtered water, but displayed a distinct population decline in unfiltered seawater (Oliver *et al.* 2006; Wollenberg & Ruby 2012), likely due to predation or competition from other seawater inhabitants. This is one of the only experiments demonstrating that symbionts have an increased reproductive capacity and higher fitness within hosts relative to non-host environments. It is important to note that this experiment found an effect because it utilized natural environments (ocean water with diverse microorganisms and nutrients) rather than culture based conditions.

Population growth is an appropriate measure of fitness for many microbes because growth and offspring production are usually the same, *i.e.* binary fission. There are many easy and reliable methods for measuring microbial population growth, including counting by culturing (CFUs or OD_{600}), counting labeled cells with a microscope or flow cytometer, and counting gene copies with quantitative PCR (qPCR). However, there are alternative measures of fitness, that include future reproduction (Baker & Romanski 2007; Dubilier *et al.* 2008; Fay *et al.* 2009; Ratcliff *et al.* 2012; Van Horn *et al.* 2012; FitzPatrick *et al.* 2012; Garcia *et al.* 2014), reproductive structures (*e.g.* fruiting bodies (Huang *et al.* 2006; Wollenberg & Ruby 2009), sporulation (Pringle & Taylor 2002; Elliott *et al.* 2009; Baker *et al.* 2013; Engelmoer *et al.* 2014), transmission (Mouton *et al.* 2004; Huang *et al.* 2006), and virulence (Bryner & Rigling 2012; Engelmoer *et al.* 2014), that can also be

employed. These measures are routinely used to measure pathogen fitness; for instance, measuring virulence as a percentage of hosts killed as a proxy for microbial fitness (Mouton *et al.* 2004; Parker *et al.* 2014). These alternative fitness measures may be more appropriate for many symbionts, especially those with complex lifecycles such as fungi (Bull & Rice 1991; Pringle & Taylor 2002) and protists (Devreotes 1989; Dunn & Weis 2009). Certain nodulated rhizobia, for example, undergo multiple rounds of endoreplication, each time doubling the chromosome without completing cell division (Wang *et al.* 2009; Laughton *et al.* 2011; Udvardi & Poole 2013). Therefore, comparing population sizes of rhizobial bacteria in and outside the host using a gene counting method like qPCR would provide an inflated count of population size and an alternative measure would be more appropriate. Additionally, alternative fitness measure may detect a benefit to symbionts even when their relative growth rate is lower in hosts than other niches.

One challenge of comparative fitness assays is duplicating an appropriate non-host environment. For example, gene expression differences between symbiotic and free-living rhizobia have been investigated in many studies, but they have almost exclusively used cell culture as the "free-living" environment (Djordjevic 2004; Barnett *et al.* 2004; Sachs & Wilcox 2006; Capela *et al.* 2006; Karunakaran *et al.* 2009; Tatsukami *et al.* 2013; Kim *et al.* 2013; Peng *et al.* 2014). Comparison between host-associated and cultured symbionts can provide insight into responses to ecologically relevant conditions, such as low-oxygen and nutrient-limitation, but they cannot duplicate the complexity and heterogeneity of natural conditions. Ideally, fitness experiments would be done in substrate taken directly from the

environment, as was the seawater for the *V. fischeri* experiment above. Semi-natural substrates like potting soil or aquarium sea salt mixtures are somewhat more informative than cell culture. In other cases, it may not be known if there is a non-host habitat or what the symbiont's full habitat range is and coupling symbiosis research with more traditional microbial ecology can inform these experiments (Zahran 2001; Nyholm & McFall-Ngai 2004; Kim *et al.* 2013; Garcia *et al.* 2014).

Advances in "omics" technologies (genomics, transcriptomics, etc.) have provided new approaches to investigate symbiont fitness. Although omics approaches do not directly test symbiont fitness, they can illuminate the "terms" of the relationship and hint at benefits. For instance, up-regulation of vitamin production in the host could suggest a nutritional benefit for symbionts, while overexpression of anti-phage proteins may indicate protection of symbionts from pathogens. Omics data can be used to direct and refine comparative fitness assays. For example, simultaneous transcriptome sequencing of *Porites* (a coral) and *Symbiodinium* (its symbiont), revealed that neither partner could synthesize a complete repertoire of amino acids. This, coupled with up-regulation of transport proteins, suggests amino acids are transported between host and symbiont, including amino acids that may be a limiting resource for *Symbiodinium* outside the host (Davidson *et al.* 2004; Login & Heddi 2013; Shinzato *et al.* 2014). Targeted experiments could test the fitness effect of nitrogen-limitation or removal of specific amino acids on *Symbiodinium* growth in the host and seawater. Omics studies may be especially useful when laboratory fitness assays do not reveal any difference

between host-associated and free-living microbes (because the benefit depends on a factor not present in the lab).

One disadvantage of growth as a fitness measure is its emphasis on short-term, immediate benefits at the expense of long-term, rare benefits, which could include access to novel genetic diversity or dispersal. Horizontal gene transfer (HGT) is an important source of novel DNA in prokaryotes, and there is considerable evidence that HGT is important in symbiosis (Marchetti *et al.* 2010; McFall-Ngai *et al.* 2013; Ratzka *et al.* 2013; Husnik *et al.* 2013). HGT is impeded by separation between appropriate donor-recipients pairs, which could be overcome when closely-related prokaryotes, which are more likely to be compatible (Popa & Dagan 2011; Login *et al.* 2011), come together in a host. HGT is particularly prevalent in proteobacteria (Udvardi & Poole 2013; Nielsen *et al.* 2014), phyla rife with insect (Wooldridge 2010; Kikuchi *et al.* 2011), marine invertebrate (Dubilier *et al.* 2008; Bright & Bulgheresi 2010; Login & Heddi 2013), and leguminous plant symbionts (Ahmadjian 1993; Zahran 2001). Genomic analysis indicates genes that control host specificity and colonization in the proteobacteria *Xenorhabdus nematophila* (Muscatine *et al.* 1984; Falkowski *et al.* 1993; Cowles & Goodrich-Blair 2008) and *V. fischeri* (Baghdasarian & Muscatine 2000; Mandel *et al.* 2009) have likely been acquired via HGT. Although some proteobacterial endosymbionts have lower rates of HGT than their close relatives (Graf & Ruby 1998; Kloesges *et al.* 2011; Macdonald *et al.* 2012), this is not true for proteobacteria in mammalian guts (McFall-Ngai *et al.* 2013). Additionally, HGT may be especially adaptive for horizontal symbionts as they could access novel DNA within hosts, even if host association was detrimental

to short-term fitness. Dispersal may be a similarly rare but beneficial event. Mobile hosts such as flying insects or pelagically dispersed coral larvae (Wirshing *et al.* 2013) may transport symbionts to novel environments or hosts that better support symbiont growth. Dispersal would be of particular benefit in systems where local extinction is possible. These rare benefits may provide small or hard-to-measure fitness gains to symbionts that outweigh other short-terms costs associated with inhabiting a host or another niche.

Finally, in order to persist, horizontal symbionts must outlive their host by dispersing to a new host or free-living habitat. In some systems, there is clear release of viable symbionts back into the environment. Bobtail squid expel ~95% of their symbionts in a daily cycle (Lee & Ruby 1994) and gene expression studies indicate symbionts prepare for life outside the host before expulsion by up-regulating flagellar genes and making metabolic changes (Jones & Nishiguchi 2006; Wier *et al.* 2010). Some legumes (Bright & Bulgheresi 2010) and marine invertebrate hosts (Sachs & Wilcox 2006), including coral (Baghdasarian & Muscatine 2000), also release viable symbionts, though this has primarily been considered a way to rid themselves of poor symbionts (Douglas 2008). In contrast, some hosts can kill, digest, or otherwise prevent viable symbionts from cycling back into the environment. Some rhizobia have undergone such extreme physiological changes that they are no longer viable outside the host, though they do remain metabolically active (Mergaert *et al.* 2006). In many systems, it is unknown whether symbionts can leave the host much less whether they are viable in the environment. Determining whether a symbiont can leave the symbiosis and proliferate is

important as transmission dynamics, the cornerstone of pathogen fitness and evolution (de Roode *et al.* 2008), undoubtedly play a role in the ecology and evolution of beneficial symbionts as well.

Symbiosis is an important and intensely studied topic in evolution and ecology. However, core concepts including how beneficial symbioses are formed and maintained over evolutionary time are not well developed. The most common hypothesis is that these associations are maintained through mutual benefit. However, in cases where there is no evidence of a symbiont benefit, symbionts may instead be more akin to prisoners or farmed crops than equal partners. Even if symbionts do exhibit increased reproductive ability in hosts, this could ultimately be of little evolutionary benefit, in much the same way cattle populations increase through ranching but, as most cattle are sacrificed prior to reproduction, they do not receive a fitness benefit. Therefore, it is important to determine whether hosts imprison symbionts and whether symbionts have adaptations to evade capture in addition to measuring costs and benefits of presumed mutualisms (Douglas 2008). Even in this warden-prisoner model of host-microbe association, it is important to recognize there may be both costs and benefits to associating with a host and to identify the short- and long-term fitness consequences for microbes in a variety of contexts. Ultimately, it is clear that progress in symbiosis research requires inclusion of the symbiont side of symbiosis.

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Chapter 5

The encapsulation immune response in pea aphids is enhanced by a secondary symbiont in a genotype specific manner

Modified from:

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ABSTRACT

Conflict over resource allocation is an inherent disadvantage in symbiotic relationships. Management of this inter-partner conflict is key to the maintenance of symbiotic relationships throughout the partners' lifecycle and over evolutionary time. The innate immune system of insects has been implicated as a conflict management tool in a number of insect-bacteria symbioses, but may be hindered by outsourcing defense against pathogen to bacterial symbionts that may provide defense by modulating host immunity. The pea aphid, *Acyrthosiphon pisum*, is a novel study system for investigating host-parasite interactions due to its complex associations with both well-characterized bacterial symbionts and a diversity of pathogens and parasites. However, little is known about the immune responses of aphids. I assessed the cellular encapsulation responses in the presence and absence of three alternative secondary symbionts, investigating the role of host and secondary symbiont genotype in encapsulation. I found that pea aphids form melanotic, but not cellular, capsules around Sephadex beads, a proxy for parasitoid wasp eggs. *Regiella insecticola* was the only secondary symbiont that enhanced the encapsulation response, and variation in this response was largely due to specific

Regiella strains. Host genotype was less influential in determining immunity outcomes. Our results highlight the importance of secondary symbionts in shaping host immunity. Understanding the complex physiological responses that can be propagated by host-symbiont associations has important consequences for host ecology, including symbiont transmission and maintenance.

INTRODUCTION

Establishing endosymbiotic relationships can confer substantial benefits to hosts, including provisioning of nutrients and protection from pathogens (see (Su *et al.* 2013) for a review). Such intimate interactions often result in fundamental changes in host physiology and phenotype either as a direct or indirect response to symbiont presence (Montgomery & McFall-Ngai 1994; Braendle *et al.* 2003). While some changes may be beneficial for the host, others that affect host life history traits such as survival and fecundity may ultimately be costly (Oliver *et al.* 2006; Simon *et al.* 2011; Vorburger & Gouskov 2011). In turn, these changes to host physiology can affect the long-term maintenance of microbial symbionts by altering the host niche that symbionts inhabit.

Inter-partner conflict over resource allocation is an inherent aspect of all symbioses, even mutualisms, but one or both partners in evolutionarily stable symbioses can evolve mechanisms to alleviate or resolve conflict to maintain the relationship. Hosts, as the larger partner with a smaller population size (Sachs *et al.* 2004), are more likely to evolve mechanisms that lead to conflict resolution or management. Components of the host immune system have been hypothesized to serve as tool of conflict management by culling or regulating symbiont populations that take excessive host's resource and risk becoming virulent (Laughton *et al.* 2014). In order to cultivate a symbiotic relationship, hosts must be able to respond differently to symbiotic and pathogenic microbes, but this can prove problematic. Microbe-associated molecular patterns (MAMPs), for example, used by hosts to recognize and respond to pathogens, are also commonly found in other microbiota,

including symbionts (Chu & Mazmanian 2013). Consequently, symbiosis may select for physiological or evolutionary changes in the host defenses that facilitate establishment and maintenance (see (Nyholm & Graf 2012) for review).

There are two ways symbiosis, especially with facultative symbionts, could affect host immunity and its potential for conflict management. First, a host may have a more active, robust, or symbiont-targeted immune system to control the symbiont population This is true in weevils, tsetse flies, and bean bugs, which all exhibit symbiont-targeted immune reactions that are induced when symbionts live within the host (Wang *et al.* 2009; Login *et al.* 2011; Kim *et al.* 2013; 2014). Alternatively, reducing or down-regulating the host immune system may promote a stable symbiont population (Gerardo *et al.* 2010; Douglas *et al.* 2011) while avoiding the self-harm associated with prolonged immune stimulation. This is especially probable in the case of facultative (secondary) symbionts, which can be acquired via horizontal transmission (Russell & Moran 2005; Henry *et al.* 2013) from other insects (Gehrer & Vorburger 2012) and may trigger an immune response after their introduction to a new host. Secondary symbionts are also more likely to come into contact with circulating hemocytes (Hinde 1971; Fukatsu *et al.* 2000) and stimulate an immune response as they are less likely to be intracellular than obligate symbionts. However, a reduced immune response may come with the cost of weakened policing of deleterious microbes, as has been shown in weevils (Vigneron *et al.* 2012).

Symbionts may also modulate the host immune response in such a way that they increase resistance to pathogens or other invaders, thereby increasing host

fitness and selection to maintain the symbiosis. Many secondary symbionts confer compensatory or additive pathogen protection upon their hosts (Montllor & Maxmen 2002; Oliver *et al.* 2003; Scarborough 2005; Haine 2008), perhaps as a way to promote their own survival by ensuring their host remains alive. *Hamiltonella defensa* in pea aphids, for example, can confer nearly complete protection against parasitoid wasps, which are otherwise frequently lethal, when it harbors a toxin-producing phage. However, recent research has shown that aphid hosts without *Hamiltonella* can protect themselves from wasp eggs, and it is unknown whether the toxin acts alone or in concert with the aphid immune system{Oliver:2009bv}. A second symbiont, *Regiella insecticola*, can also provide protection against parasitoid wasps in the peach-potato aphid (Vorburger *et al.* 2010), possibly through a toxin or other pathogenicity factors though the mechanism is less clear (Hansen *et al.* 2012).

In insects without these endosymbionts, hosts protect themselves from wasp eggs and other invaders too large to be phagocytized through encapsulation (Dunn 1986; Lackie 1988), a defense in which multiple hemocytes bind to the foreign object and form an overlapping sheath of cells that sometimes also melanize (Pech & Strand 1996; Gillespie *et al.* 1997) (Lavine & Strand 2002). Some encapsulation responses do not produce cellular capsules but instead only melanize the foreign target in the absence of hemocytes to form a melanotic capsule (Lavine & Strand 2002; Strand 2008). In both cases, the foreign object is killed or degraded within the capsule by small molecules, possibly reactive oxygen species (Lemaitre & Hoffmann 2007; Dubovskii *et al.* 2010), phenoloxidase {Kato 2014}, or symbiont-produced molecules.

In order to understand how secondary symbionts might alter the encapsulation response, we used the pea aphid, *Acyrthosiphon pisum*, as a model system. The pea aphid has greatly reduced or altered immune responses compared to other invertebrates studied to date (Altincicek *et al.* 2008; Gerardo *et al.* 2010), and it is hypothesized to protect itself largely through compensatory mechanisms, such as outsourcing defense against pathogens to symbionts as discussed above. Here we look at the effect of secondary symbionts on encapsulation, and investigate the role of host and secondary symbiont genotype in specific responses. First, we visualize and measure cellular encapsulation and melanization over a time course typical of encapsulation in other insects (Lavine & Strand 2002), allowing us to understand the baseline encapsulation response of the aphid in its simplest symbiotic state (hosting the obligate symbiont, *Buchnera*, but no secondary symbionts). Second, I test the effect of three prevalent secondary symbionts, *Hamiltonella defensa*, *Serratia symbiotica*, and *Regiella insecticola*, on the encapsulation response in three clonal aphid lines, each singly infected with one secondary symbiont, and one no secondary symbiont control line. Finally, I investigated the effect of both aphid genotype and secondary symbiont genotype on the encapsulation response using different strains of *Regiella*, which produced the strongest effect on encapsulation.

MATERIALS AND METHODS

Aphid genotypes and rearing conditions

All assays were carried out on clonally produced female offspring from parthenogenetic females of two genetically distinct pea aphid genotypes. The 5A line was collected in 1999 from Madison, WI and contained only the obligate symbiont, *Buchnera* (5A0) with no other known secondary symbionts (Moran *et al.* 2005). Three different secondary symbiont species were introduced into 5A0 aphids in 2003 via hemolymph transfer from secondary symbiont-hosting donor aphids, producing clonal lines that harbor single infections of *Serratia symbiotica* (5AR), *Hamiltonella defensa* (5AT), or *Regiella insecticola* (5AU; (Oliver *et al.* 2003). The LSR1 genotype, collected near Ithaca, New York in 1998, was used to further investigate the role of *Regiella* in encapsulation. This genotype was naturally infected with a *Regiella* strain, Ri, and was subsequently cleared with antibiotics to produce the LSR1 no symbiont control (Douglas *et al.* 2006). For the final experiment, the 5A and LSR1 aphid genotypes were separated into four different clonal lines: a no symbiont control line (contained no secondary symbionts), and three lines each harboring one of three different strains of *Regiella*: 5.15, Ri, and U. Strain 5.15 was collected from a peach-potato aphid, *Myzus persicae*, in 2003 at Bucchus Marsh, Australia, and has previously been shown to protect against parasitoid wasps (Vorburger *et al.* 2010; Hansen *et al.* 2012). Strain U was specific to the 5A aphid genotype, having been introduced as a new infection 10 years previously (see above). To produce a fully factored cross infection of the two aphid genotypes with the three symbiont strains, U was introduced into the LSR1 aphids, Ri into the 5A aphids, and 5.15 introduced into both host genotypes. Transfer of the symbionts was carried out via injection of donor hemolymph at least one year prior

to the experiment (Hansen *et al.* 2012). Aphids were kept in cages in a walk-in growth chamber and maintained on *Vicia faba* (fava bean) at 20 ^{o}C and a 16 hr light:8 hr dark cycle.

Encapsulation Assays

I assessed whether pea aphids are capable of forming cellular or melanotic capsules by injecting Sephadex beads (40-125 μm) dyed with Congo red into 12-day old aphids above the right siphunculus using a glass needle. Sephadex beads are commonly used to measure the encapsulation response in insects as a proxy for naturally occurring foreign objects such as parasitoid eggs (Smilanich *et al.* 2009). Aphids were allowed to recover for 15 minutes and were then placed on fresh plants and reared under the normal conditions above. The red-dyed Sephadex beads were removed via dorsal dissection after an incubation period of 12, 24 or 96 hr. Retrieved beads were added to 20 μl of Carlson's solution (Mitsuhashi 2002), a buffer for viable insect cells, on a teflon ringed slide and viewed under phase contrast optics. Non-injected control beads were also added to a well with 20 μl of Carlson's solution to serve as a no encapsulation/no melanization negative control. The degree of encapsulation and melanization, if present, was quantified by measuring the red (*r*) value of each experimental and negative control bead averaged over a 101 x 101 pixel area in Photoshop CS3. The *r* value is a numerical measure of the redness of the retrieved experimental beads, which was normalized to the r value of the baseline negative control beads; since melanin is dark brown and obscures the red bead, a lower *r* value in experimental beads represents a

greater level of melanization (Smilanich *et al.* 2009). Baseline *r* values were calculated for each aphid as the mean *r* value from three different control beads. *R* values were then transformed into percentage melanization for each bead using the formula (1-(experimental *r* value/baseline *r* value) * 100) for ease of comparison (Smilanich *et al.* 2009).

I performed three encapsulation experiments that each further investigated results from the previous experiments. First, I measured the encapsulation response over time in 5A0 aphids, a line with no secondary symbionts. Then tested the effect of three alternative secondary symbionts on the encapsulation response in 5A0. Finally I measured encapsulation in two aphid genotypes harboring alternative strains of one symbiont. Each experiment is detailed below. All assays were carried out on adult female aphids that had completed their final molt and begun clonally reproducing two days prior to the experiment. All experiments required that aphids be destructively sampled during data collection. Therefore, in experiments following immune responses over a time-course, a different cohort of aphids was sampled at each time point rather than collecting multiple samples from the same individual.

Experiment 1: Because the encapsulation response is not immediate and can change over a period of days (Lavine & Strand 2002), I first measured encapsulation at three time points. Additionally, measuring the encapsulation response in an aphid line with no secondary symbionts allowed this measurement to serve a baseline encapsulation response to which the effect of secondary symbionts on encapsulation could be compared. I injected 5-15 red-dyed beads per aphid and retrieved the beads 12, 24, or 96 hrs later (the number of retrieved beads

varied between 1 and 11, average = 5). Due to time constraints associated with the assay, samples were collected over several different dates. A total of eight aphids were collected per time point.

Experiment 2. I tested the impact of three different secondary symbiont species, *H. defensa*, *R. insecticola*, and *S. symbiotica*, on the encapsulation response using the four clonal 5A lines each with a single symbiont infection or no secondary symbiont. (n = 4 - 8 aphids per line). One to twenty-three beads (mean = 7.5) were injected per aphid.

Experiment 3. Following the results of Experiment 2, I compared the encapsulation response in two aphid genotype backgrounds (5A and LSR1), comparing the no symbiont control lines with clonal lines containing single infections of each of three different *Regiella* strains (5.15, Ri and U, see above for details). One to six beads (mean = 2.8) were inserted per aphid. Due to time constraints, samples were collected over four replicate experiments with three to five aphids harvested in each replicate (final n = 14 - 20 aphids per line).

Statistical analysis

All data were analyzed using the statistical package R v2.13. The encapsulation assays were analyzed using ANOVA with the number of beads retrieved per aphid, experimental replicate, and aphid genotype included as cofactors. For the first experiment in aphids without secondary symbionts, the encapsulation data were analyzed using a nested ANOVA, with number of beads nested within each aphid. Samples within each of the paired clonal lines were also

analyzed using a Wilcoxon rank-sum test to directly compare the effect of secondary symbiont presence within each different host background. Minimal models were derived by removing terms followed by model comparisons. Terms were retained if their removal significantly reduced the explanatory power of the model.

RESULTS

Pea aphids exhibit melanotic, but not cellular, encapsulation

Pea aphids readily formed melanotic capsules around Sephadex beads as evidenced by the accumulation of melanin on the surface of beads in the absence of any bound hemocytes (Fig. 5-1A-D). In contrast, I never observed any hemocytes bound to the Sephadex beads I recovered from aphids. Most aphids formed melanotic capsules around a majority of the beads injected into them, with usually only one or two beads recovered from each aphid exhibiting no melanization. In total, only 13 out of the 107 beads recovered from the aphids showed no accumulation of melanin, with most of these originating from a single individual who did not melanize any beads. Beads closer to the site of injection tended to be more heavily melanised than beads recovered elsewhere in the hemocoel (Fig. 5-1A-D), suggesting that bead melanization may be influenced in part by proximity to the damaged cuticle of the aphid. Heavily melanized beads also appeared partially degraded (Fig. 5-1D), which may be due to enzymes from the aphid digesting the glucan polymers of the Sephadex beads. I assessed whether intensity of bead melanization varied among individual aphids, or with the number of beads injected

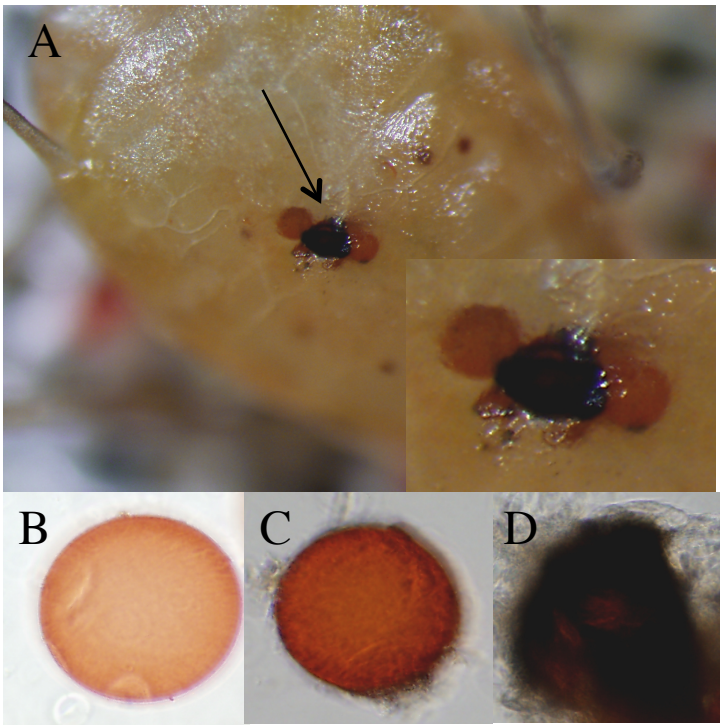

Figure 5-1. Melanization of Sephadex beads injected into aphids. (A) Low magnification image showing three melanized beads in the aphid hemocoel (arrow). A higher magnification image of the same beads shows the variation in intensity of melanisation of each bead. (B) Control bead stained with Congo Red. (C) A representative bead recovered from an aphid that was modestly melanized. (D) A representative bead from an aphid that was strongly melanized.

per aphid and collection time by measuring the red (*r*) value of recovered beads (Fig. 5-2). *R* values did not differ among the three time points (12, 24, 96 hr) at which we collected beads from aphids (ANOVA, $F_{2, 18} = 3.319$, $p = 0.0593$), the number of beads recovered per aphid (nested ANOVA, $F_{1, 18} = 1.286$, $p = 0.272$), or the date of assay (nested ANOVA, $F_{1, 18} = 2.767$, $p = 0.114$). However, I did detect a significant difference in *r* values between individual aphids (nested ANOVA, $F_{1, 18} = 11.308$, $p < 0.01$), which indicated that some aphids melanised beads more strongly than others.

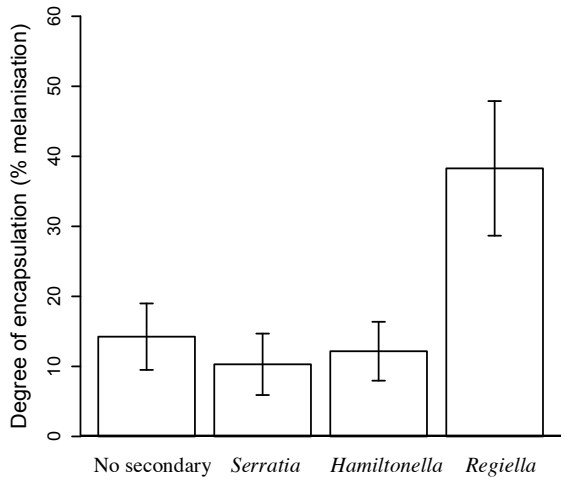

Figure 5-2. Degree of encapsulation, calculated as the average percentage melanization of Sephadex beads per aphid at 12, 24 and 96 hours after injection, relative to unmelanized (red) control beads. A value of 0% indicates no melanization while a value of 100% indicates intense melanization sufficient to obscure detection of any red color (error bars ±1 s.e.m, n = 8 per time point).

Regiella *symbiont increases the encapsulation response*

There was a significant effect of symbiont line on encapsulation response (ANOVA, $F_{3,19} = 3.65$, $p = 0.031$), with the *Regiella*-infected aphids exhibiting a stronger encapsulation response than either the control or other secondary symbiont lines (Fig. 5-3). There was no significant effect of bead number (ANOVA, $F_{1,18} = 1.84$, $p = 0.191$) on encapsulation response.

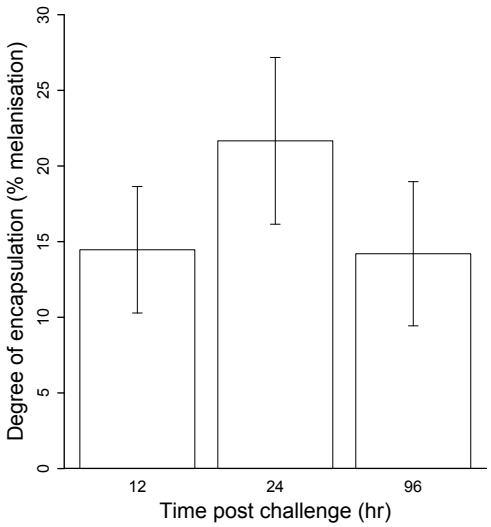

Figure 5-3. Degree of encapsulation in the 5A aphid clonal lines, comparing the no secondary symbiont control with lines containing single infections of *Serratia*, *Hamiltonella* and *Regiella* symbionts.

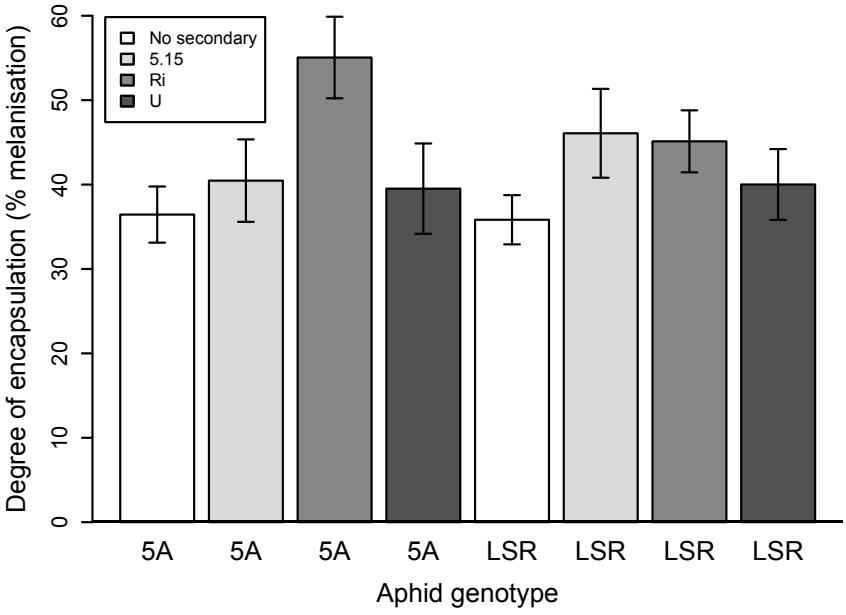

Figure 5-4. Degree of encapsulation in two aphid genotypes (5A and LSR1), each split into four clonal lines: no secondary symbiont (control) lines and three lines each containing a single infection with one of three *Regiella* symbiont strains (error bars ±1 s.e.m).

Regiella *strains vary in the degree of improvement in the encapsulation response*

The presence of a *Regiella* symbiont significantly increased the encapsulation response (Fig. 5-4, ANOVA, $F_{1,130} = 5.25$, $p = 0.024$), with the magnitude of the effect varying significantly depending on the *Regiella* strain (ANOVA, $F_{3,137} = 3.48$, $p = 0.018$). Specifically, Ri presence induced a greater encapsulation response than the other *Regiella* strains (Fig. 5-4). There was no significant effect of the number of beads per aphid (ANOVA, $F_{5,130} = 0.94$, $p = 0.457$), experimental replicate (ANOVA, $F_{3,130} = 1.76$, $p = 0.159$) or host genetic background (ANOVA, $F_{1,130} = 0.23$, $p = 0.632$) on the encapsulation response.

DISCUSSION

Our results indicate that pea aphids do not form cellular capsules but do produce melanotic capsules around Sephadex beads. From these results, I cannot conclude that pea aphids are incapable of forming cellular capsules, although recent data indicate that pea aphids also fail to form cellular capsules around other foreign objects (Schmitz *et al.* 2012) and eggs from the parasitoid *Aphidius ervi* (Strand, M. R. and Oliver, K. M., unpublished observations). Two factors potentially contribute to these outcomes. First, hemocyte abundance often positively correlates with the ability of insects to form cellular capsules (Siva-Jothy *et al.* 2005). I estimate that hemolymph from adult pea aphids contains on average 1,800 hemocytes μl^{-1} (prohemocytes + granulocytes + an estimated 9% representation of oenocytoids). In contrast, hemocyte densities for other hemimetabolous insects that form cellular

capsules are much higher. For example, the American cockroach, *Periplaneta americana*, contains ~80,000 haemocytes μl^{-1} (Wheeler 1963), while the bark bug *Halys dentate* contains ~13,000-15,000 haemocytes μl^{-1} (Bahadur & Pathak 1971). A second contributing factor may be the absence of a hemocyte type specialized for capsule formation. In Lepidoptera, capsules are primarily formed by plasmatocytes, whereas *Drosophila* employs capsule-forming hemocytes called lamellocytes (Lanot *et al.* 2001; Lavine & Strand 2002; Eslin & Doury 2006; Strand 2008). In contrast, the mosquitoes *Aedes aegypti* and *Anopheles gambiae* produce phagocytic granulocytes and melanin-producing oenocytoids but like pea aphids lack a specialised capsule-forming haemocyte and mount only a melanotic encapsulation response (Castillo *et al.* 2006). Our observation of degradation when beads are more heavily melanised may reflect another defense response or the effects of the cytotoxic by-products formed during the production of melanin (Söderhäll & Cerenius 1998). The pea aphid encodes two pro-phenoloxidase genes (Gerardo *et al.* 2010) but whether either is expressed in oenocytoids remains unknown.

Only one of the secondary symbionts tested, *Regiella insecticola*, drastically enhanced the encapsulations response in the pea aphid. When the host background was kept constant (i.e. in the 5A aphids), encapsulation responses were only ever increased by *Regiella* presence. *Regiella* in the 5A aphid line has been found to have a lower virulence, including a lower rate of replication, than other secondary symbionts (Laughton *et al.* 2014) and therefore may not stimulate host immune activation to the same extent, allowing host resources to be focused on defense, such as the encapsulation response. Infection with secondary symbionts may also

differentially improve the nutritional status of the host, or indirectly increase host vigor via other mechanisms (Leonardo & Muiru 2003; Tsuchida *et al.* 2004; Scarborough 2005; Ferrari *et al.* 2007; Hansen & Moran 2014), providing hosts with the resources to produce a universally heightened immune response. However, to date, the mechanisms underlying these phenotypic variations are unknown and evidence for beneficial effects appear to be context dependent (Ferrari & Vavre 2011).

It could be reasonably predicted that since *Regiella* augments encapsulations it will provide the best protection against parasitoid wasps, but parasitism trials have shown that is not the case. In the pea aphid, *Hamiltonella* and *Serratia* are the only symbionts have been shown to defend against parasitoids (Oliver *et al.* 2003; 2006) (Oliver *et al.* 2005) though *Regiella* is effective in another aphid species, *Myzus persicae* (Vorburger *et al.* 2010). Additionally, the strain that enhanced melanisation the most here, Ri, has no protective effect in two other aphid species (Vorburger *et al.* 2010). It may be that melanization is not an important aspect of protection against parasitoids or that melanization is not effective on wasp eggs compared to the antigenically-inert Sephadex beads. It is important to note that recent findings show aphid-encoded protection is more prevalent than previously thought and the 5A and LSR lines have two of the lowest levels of protection from parasitoids in the absence of symbiont-encoded protection and therefore may have low levels of melanization in general (Martinez *et al.* 2014). However, it may not be the level of melanization that is important, but the way it interacts with the symbiont-encoded protection. For example, *Hamiltonella* cells must be lysed by the

protective phage to release the phage-encoded toxin, which could be one of the degradation factors released after the melanotic capsule is formed. This hypothesis is supported by the observations that *Hamiltonella* can circulate freely in the hemolymph (Moran *et al.* 2005) and are seen internally and externally associated with hemocytes (Schmitz *et al.* 2012).

Focusing on the effect of specific symbiont species, our results showed significant variation in the encapsulation response based on the *Regiella* strain. Such genotypic variation is increasingly found to be important in shaping host-symbiont interactions with regards to the benefits that secondary symbionts can confer to their hosts (Ferrari *et al.* 2007; Castañeda *et al.* 2010; Vorburger *et al.* 2010; Oliver *et al.* 2014). In both *Regiella* and *Hamiltonella*, the protection phenotype ability varies based on symbiont strain and host aphid species (Oliver *et al.* 2003; Vorburger *et al.* 2010; Hansen *et al.* 2012). The *Regiella* strains also exhibited variations in altering the encapsulation response (the 5.15 and Ri *Regiella* strains were the same as those used by (Hansen *et al.* 2012)). The extent of the differences seen in the immune responses I assayed is therefore perhaps not surprising considering the potential for genetic diversity within these seemingly similar systems (Schmitz *et al.* 2012). Such differences could have wide-reaching consequences for symbiont and host phenotypes, including potential effects on host immune responses.

This study highlights the importance of secondary symbionts in shaping host immunity. Our findings indicate that while variation in magnitude of effect is present, *Regiella* reliably stimulates the melanotic encapsulation response. Further

work is needed to identify the mechanisms involved in these triggers and tradeoffs, with previous research hinting at a complex interaction of factors including symbiont replication rate and virulence effects (Oliver *et al.* 2009; Hansen *et al.* 2012; Laughton *et al.* 2014). Understanding these interactions, and the magnitude of genetic variation attributed to secondary symbionts, has important consequences for host health, and symbiont pathogen maintenance and transmission dynamics.

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Chapter 6

Conclusions and Future Directions

Symbionts, or microbial mutualists, can have profound effects on the ecology and evolution of multicellular organisms. These effects can differ in type or magnitude based on genetic and phenotypic variation in symbionts and so it is important to determine and understand the consequences of specificity in host-symbiont pairings. In this thesis, I have investigated symbiont specificity in vertical and horizontal symbioses within a framework of symbiont acquisition and maintenance and measured the impact of selective maintenance on host ecology and evolution in a vertical symbiosis. The impact of each of acquisition and maintenance on the two investigated symbioses -- that between true bugs and *Burkholderia* bacteria and between pea aphids and various facultative bacterial symbionts -- highlights the need to take a comprehensive approach when studying symbiosis.

Chapter 2: Considerations and Future Directions

Host-symbiont pairings exhibit a range of specificity from specialist associations in which a host or symbiont can only pair with one or a limited range of partners to generalist associations in which either the host or the symbiont can have many partners (Baker 2003). Variation in specificity is especially evident in

horizontal symbioses as hosts can either acquire a diverse subset of microbes from the external environment (Dillon & Charnley 2002; Van Horn *et al.* 2012; FitzPatrick *et al.* 2012) or they can select a very narrow subset of those microbes through host screening processes (LaJeunesse *et al.* 2004; Wentrup *et al.* 2013). It is not yet clear how ecological conditions and evolutionary selection shape placement on this continuum.

Many stinkbugs harbor a limited diversity of *Burkholderia* symbionts in the midgut crypts, a specialized symbiont-bearing organ found across the Lygaeoidea and Coreoidea superfamily of insects, but it is unknown how this specificity is determined. In chapter two, I investigate the specificity of *Burkholderia* associations in four sympatric broad-headed bug species, assessing how the bug symbionts relate to the environmental pool of bacteria and how host species and geography impact symbiont associations. I found that host screening processes are likely similar across host species as there was one *Burkholderia* strain that was prevalent across all four hosts. Previous work in Asian stinkbugs suggests host screening is in part mediated by immune reactions in or near the crypts, but the most prevalent *Burkholderia* in these insects differed from the most prevalent *Burkholderia* in broad-headed bugs and it is unknown if similar mechanisms could select for different *Burkholderia* species. Future work could include reciprocal cross-infection of different hosts with both *Burkholderia* species to determine whether host screening in each species can act in a similar manner on other *Burkholderia*.

Interestingly, I also found genetically similar *Burkholderia* were hosted in broad-headed bug midguts and nitrogen-fixing root nodules of bush clover, the

primary food source of broad-headed bugs. Although *Burkholderia* association is not commonly present in bush clover, it has been found in other bush clover populations suggesting it is more than a fluke occurrence (Palaniappan *et al.* 2010). This strain provides the opportunity to investigate the factors that influence host specificity in *Burkholderia* using comparative genomics to determine genes unique to each strain or using reciprocal cross-infections with random mutagenesis to identify factors that promote colonization in the plant, the insect or both.

Chapter 3: Considerations and Future Directions

In chapter three, I turn to processes of symbiont specificity in a different true bug species, the squash bug *Anasa tristis*. I surveyed the bacteria present along the five sections of the midgut, the acquisition route to the crypts, of the squash bug to determine where specificity is imposed, with a specific focus on *Burkholderia*.

There was a limited diversity of *Burkholderia* both in the crypts and in the midgut anterior to the crypts, suggesting that symbiont specificity is not unique to the crypts and is likely imposed in the host before the midgut. Screening for symbiont specificity could occur in the external environment (*i.e.* within-host screening may not be necessary if the dominant midgut strain of *Burkholderia* is also dominant in the external environment) or in the digestive system anterior to the midgut. Both of these sites need to be investigated for their role in determining bacterial specificity in the midgut. *Burkholderia* should be quantified in the squash bug habitat including the soil and food plant surfaces and phloem to determine its

relative abundance in the external environment. The rostrum (mouthpart with two stylets), salivary glands, and foregut are involved in digestion and anterior to the midgut, and should be investigated as potential screening sites. It is possible that there is no active selection mechanism imposed by the host, but instead the dominant *Burkholderia* strain is better adapted to the host niche and outcompetes other *Burkholderia* strains shortly after uptake by the host. Further work should explore the role of within-host competition between *Burkholderia* strains and other bacteria in establishment within the host niche.

Previous work in in another true bug, *Riptortus pedestris*, suggests that host screening mostly occurs in, or in regions just adjacent to, the crypts and includes both screen-in and screen-out mechanisms. For example, *Burkholderia* must exhibit certain traits to be "screened-in" to the crypts
(Ohbayashi *et al.* 2012; Kim *et al.* 2013b; a), but other bacteria are likely "screened out" when the crypts increase antimicrobial activity just before symbiont acquisition (Kim *et al.* 2014). However, my sequencing results from the squash bug midgut suggests that specificity has been established long before *Burkholderia* enter the crypts and future work should reconcile these findings. There may be different screening mechanisms in different true bug species or both pre-midgut screening and crypts screening could be part of a series of filters symbionts must pass through to reach their final destination.

Finally, this study was limited to adult squash bugs, which may give the false impression of long established bacterial specificity throughout the midgut. In another stinkbug, *Riptortus pedestris*, the acquisition of *Burkholderia* is largely

limited to the second instar developmental stage (Kikuchi *et al.* 2011), and it may be in this stage or elsewhere during development where specificity is imposed. For example, the midgut may initially be colonized by a number of *Burkholderia* strains and other bacteria, but only one *Burkholderia* strain can survive ecdysis, during which the lining of the midgut is sloughed off and replaced, disrupting or suppressing the colonization of bacteria in the midgut (Kim *et al.* 2014). This could be rectified by surveying bacterial populations in juvenile squash bugs collected from natural populations or by simultaneously infecting juvenile squash bugs with multiple *Burkholderia* strains in the lab and quantifying titers of each strain throughout development.

Chapter 4: Considerations and Future Directions

While many benefits of host-symbiont interactions have been elucidated in hosts (Kikuchi *et al.* 2007; Salem *et al.* 2014), little work has considered the costs and benefits of symbiosis for microbial symbionts (Wollenberg & Ruby 2012), and, like the fitness effects for the hosts, these costs and benefits could shape the evolutionary maintenance of symbioses. Chapter four outlines how investigating symbiosis from the symbiont's perspective can aid in understanding the evolutionary stability of mutualism and details several approaches to quantifying these costs. While many of the approaches are challenging with vertically-transmitted, host-adapted symbionts such as those studied in pea aphids (Chapter 5), many of these approaches are applicable to the true bug-*Burkholderia*

system (Chapters 2 and 3) because symbionts and hosts can be readily decoupled and studied in isolation as well as in association. Experiments could include quantifying *Burkholderia* fitness in the soil compared to within the host, and then investigating potential trade-offs in adaptations to each niche, investigating potential benefits produced for *Burkholderia* by the host through transcriptomics or metabolomics of symbiotic and aposymbiotic hosts, and producing symbiosis defective hosts through mutagenesis to understand factors that support *Burkholderia* colonization and growth in the host midgut.

Chapter 5: Considerations and Future Directions

The ubiquity of animal-microbe symbioses, many of which have persisted over long evolutionary time periods, suggests that selection is acting to maintain these associations. One way vertical symbionts may promote their own maintenance within the host is by ensuring their host lives long enough to reproduce and transmit the symbionts to the next host generation. Recent work has indicated that many symbionts do this is by protecting their hosts from natural enemies (Oliver *et al.* 2003; Scarborough 2005; Oliver *et al.* 2014) and such protection could in part be mediated by the symbionts strengthening host immune responses. I explored this possibility in the pea aphid system by measuring encapsulation and melanization, an immune defense against large foreign bodies such as parasitoid wasp eggs, in the presence and absence of alternative facultative symbiont species. One symbiont, *Regiella insecticola*, augmented the melanization

response in two clonal pea aphid lineages, though different *Regiella* strains varied in their ability to augment melanization.

Future studies should reconcile the findings from this chapter, that *Regiella* can enhance melanization, with the previous findings that *Regiella* is generally not protective against parasitoid wasps in pea aphids (Oliver *et al.* 2003; Vorburger *et al.* 2009), though it is in peach-potato aphids (Vorburger *et al.* 2010), and the fact that two other facultative symbionts, *Hamiltonella* and *Serratia,* provide protection to pea aphids (Oliver *et al.* 2003; 2006), but did not enhance the melanization response. Firstly, the effect of *Regiella* strains on melanization should be quantified in the peach-potato aphid, the aphid species in which it confers protection, to test for a correlation between increased melanization and increased host survival. This would be a good first test of whether enhanced melanization plays a role in defense against parasitoid wasps and, ultimately, host survival. Secondly, there is clearly variation in the ability of *Regiella* to modulate a host's response to parasitoid attack and further studies could systematically quantify this variation across the full breadth of *Regiella* and host genotypes to understand the basis for this variation. A larger study could also test for symbiont genotype x host genotype interactions, a possibility hinted at in my experiments, which likely had sample sizes that were too small to detect an interactive effect.

Summary

Overall, this work provides novel insights into processes shaping both horizontally and vertically transmitted, beneficial symbioses. I have laid the foundation to continue to develop *Anasa tristis* as a model to study the roles that environmental and host screening play in symbiont establishment. I have also provided evidence that beneficial symbionts can impact host immune function, which has implications for how host may receive protection from symbionts against natural enemies and for the complex interplay between host immunity, microbial friends, and natural enemies. The diverse approaches here, coupling phylogenetics, deep sequencing, and immunological assays highlight the challenges of developing a comprehensive understanding of the acquisition and maintenance of the world's diverse symbioses.

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